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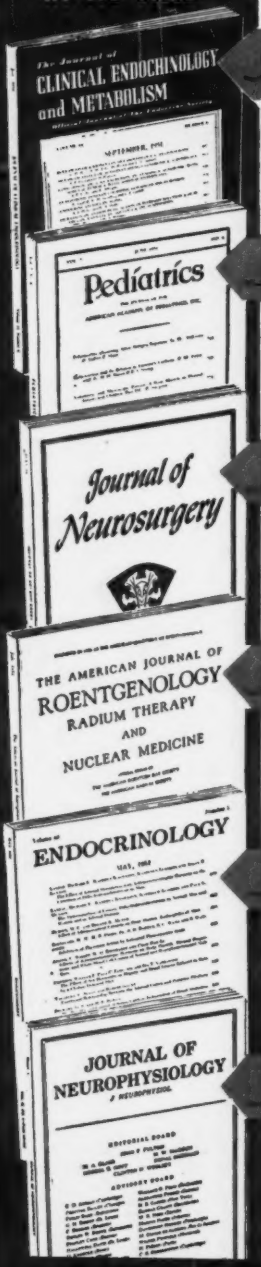

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THE ADRENAL CORTEX OF THE PANTOTHENIC ACID-DEFICIENT RAT: EOSINOPHILE AND LYMPHOCYTE RESPONSES[1,2]

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INTRODUCTION

THE importance of pantothenic acid in the maintenance of the adrenal cortex of the rat has been known on morphological grounds since the initial observations of Daft and Sebrell (1939) Nelson (1939), Daft *et al.* (1940), and Ashburn (1940). More recently the histological changes occurring in the adrenal of the pantothenic acid-deficient rat have been reported in some detail by Deane and McKibbin (1946), who have described such changes as hypertrophy, depletion of sudanophilic and "ketosteroid" substances, and focal necrosis, hemorrhage and fibroblastic activity. As noted by previous observers, these changes appear to affect predominantly the zona fasciculata and the zona reticularis.

Surprisingly few functional studies of the adrenal cortex of the pantothenate-deficient rat have been reported. Gaunt *et al.* (1946) have shown that deficiency of this vitamin increases the sensitivity of the rat to water intoxication as compared to pair-fed controls. This increased sensitivity could be abolished, however, if the animals were pretreated with adrenal extract, desoxycorticosterone or calcium pantothenate.

Compared with control rats, the pantothenic acid-deficient rat shows a less marked lymphopenia following either forced swimming or ACTH

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[1] This investigation was supported in part by grants from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

[2] We are indebted to Dr. Irby Bunding of Armour and Company for gifts of ACTH; to Merck and Company for cortisone; and to Hoffmann La-Roche and Abbott Companies for vitamins.

administration (Dumm *et al.*, 1949). Restitution of more normal function could be obtained by allowing the animals pantothenate for a short period and then retesting them.

The picture with regard to salt metabolism is somewhat confusing. Supplee *et al.* (1942) have reported that the pantothenic acid-deficient animal prefers a higher salt intake than control animals when placed on a self-selection dietary regimen. However, studies designed to demonstrate a salt-wasting syndrome in the deficient animals when placed on a diet low in sodium have been unsuccessful (McQueeney *et al.*, 1947).

The present paper reports the results of a study designed to ascertain the functional integrity of the adrenal cortex of the pantothenic acid-deficient rat using the eosinophile and lymphocyte responses as indices.

METHODS

Animals and diets. All animals in this study were males of the Sprague-Dawley strain. As received in the laboratory at 23–25 days of age these animals had an average

TABLE 1. COMPOSITION OF PANTOTHENIC ACID-DEFICIENT AND CONTROL DIETS

Constituents	Per cent
Vitaminized Casein[1]	10.0
Labco (vitamin-free) Casein	15.0
Vitaminized Corn Oil[2]	5.0
Salts IV[3]	4.0
Choline	0.1
Cystine	0.2
Sucrose	67.0

[1] Vitaminized casein when added in above amount contained the following amounts of vitamins per 100 grams of diet:

Thiamin HCl	1.0 mg.
Riboflavin	1.0 mg.
Pyridoxine	1.0 mg.
Nicotinic acid	10.0 mg.
Calcium pantothenate (deleted in deficient diet)	10.0 mg.
Biotin	100.0 μg.
Folic acid	100.0 μg.
Inositol	20.0 mg.
Paraaminobenzoic acid	20.0 mg.

[2] Vitaminized corn oil when added in above amount contained the following amounts of vitamins per 100 grams of diet:

Alpha-tocopherol acetate	10.0 mg.
Vitamin K	500.0 μg.
Vitamin D_3	10.0 μg.
Vitamin A	3000. I.U.

[3] Salts IV used was the mixture of Phillips and Hart (1935) modified by the addition of 0.03% $CoCl_2 \cdot 6H_2O$.

weight of 45 grams. The animals were housed in individual screen-bottom cages in a room where the temperature and the humidity were held reasonably constant at 25° C. and 50–55% respectively. Tap water was supplied *ad libitum*. The composition of the pantothenic acid-deficient and control diets are shown in table 1. The deficient diet was fed *ad libitum*, while the control animals were restricted to amounts of food approxi-

mately equal to that consumed by the deficient group. Experiments were done on the animals after they had subsisted on the deficient and control diets for five weeks. At this time, the average weight of the controls was 120 grams and of the deficients was 90 grams. It should be mentioned that a severe degree of inanition does not characterize this deficiency syndrome, as it does so prominently in certain others. This observation has also been reported by others (Ralli, 1949; Voris *et al.*, 1942). No animal was chosen for experiment unless free of respiratory infection.

Hematological. Free flowing tail blood was used for all cell counts. Eosinophile counts were made by the technic described by Randolph (1944), with the exception that the methylene blue was omitted from the staining fluid. In making these counts, all of the precautions recommended by McDermott *et al.* (1950) have been followed. Total white and differential counts were made by the usual technics, following the precautions as set forth by Wintrobe (1946). At least 250 cells were counted on each Wright stained film. Lymphocyte counts were calculated from total and differential counts.

The results to be presented below represent data obtained on the same series (six controls and six deficients) of animals. All animals were in the fed state at the time of the experiment, although food was removed from the cages during the experiment (4 hours in every case). After each experiment, the animals were allowed a 3–4 day period of recuperation before further experiments were undertaken. As judged by the subsequent resting eosinophile counts, this was entirely adequate.

Drugs. Epinephrine hydrochloride (Sharp and Dohme) was used. This material was diluted appropriately with isotonic saline and was administered subcutaneously in a dose of 0.02 mg. per 100 gm. body weight (Cori and Cori, 1930). Adrenocorticotrophic hormone (ACTH) was a product of the Armour Laboratories; this material had an estimated potency of approximately 8/5 of the Armour Standard ACTH (La-1-A). ACTH was made in isotonic saline and was administered by the intraperitoneal route in a dose equivalent to 1.0 mg. of the Armour Standard per 100 gm. of body weight. Cortisone used in this study was a saline suspension of cortisone acetate (Merck); it was given intraperitoneally at a dosage level of 1.0 mg. per 100 gm. of body weight.

EXPERIMENTS AND RESULTS

Response to epinephrine. In proper doses, epinephrine administered to normal rats is known to promote an eosinopenia by an endocrine mechanism involving stimulation of the anterior pituitary with the release of ACTH and the subsequent stimulation of the adrenal cortex to an increased rate of secretion of an 11-oxysteroid hormone. This test has been advanced by Thorn and Forsham (1949), for the determination of an intact and functional pituitary-adrenal cortical mechanism. The epinephrine test, therefore, seemed a good method for approaching the problem of function of the adrenal cortex of the pantothenic acid-deficient animal. Counts were made at zero and four hours after epinephrine, since this interval appears to be optimal for the detection of a maximum eosinopenic response (McDermott *et al.*, 1950; Recant *et al.*, 1950).

The response of these two blood elements to epinephrine is shown in figure 1 and table 3. Following McDermott, *et al.* (1950), the data have been plotted as percentage change in eosinophiles using the resting

value (zero hour) as 100 per cent. That there are no significant differences between the resting values of the control and deficient animals may be seen from the data in table 2. Data for the lymphocytes are expressed both as percentage change and as absolute change.

These data show a striking difference between the response of the eosinophiles of the two groups. The control animals show the usual

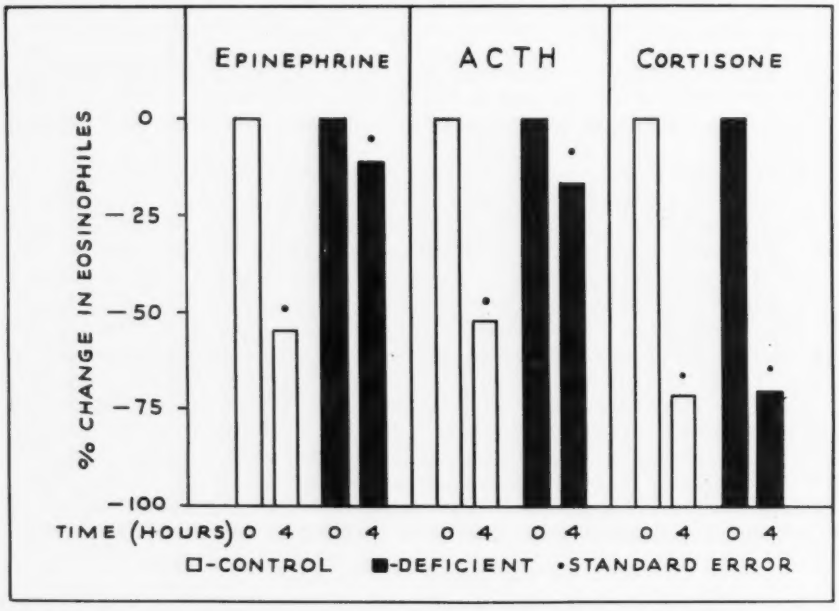

FIG. 1: The eosinophile response of pantothenic acid-deficient and control rats to epinephrine, ACTH, and cortisone administration. All control responses show statistical significance ($p = <0.01$), while only the deficient response to cortisone shows such significance.

normal response (mean fall of greater than 50 per cent) while the deficient animals show a fall of only 11 per cent. The changes in the lymphocytes tend to follow those observed in the eosinophiles, although they are not as marked and tend to be more variable. It is our impression that the changes in the eosinophiles are probably the most accurate and sensitve of the two indices. This is probably due in part to the greater accuracy of the method for enumerating eosinophiles.

Response to ACTH. Use of the epinephrine test is justified only to determine whether or not a functional insufficiency is present in the

TABLE 2. A COMPARISON OF THE RESTING LEVELS OF LYMPHOCYTE AND EOSINOPHILE COUNTS OF PANTOTHENIC ACID-DEFICIENT AND CONTROL RATS

Group	No. observations	No. animals	Lymphocytes*	Eosinophiles*
Deficient	18	6	9930 ± 590	141 ± 5
Control	18	6	9490 ± 441	147 ± 5

* Data expressed as mean of number of cells per mm.^{3} with S.E.

pituitary-adrenal cortical axis. Data from the use of this test coupled with similar data obtained from the measurement of the response to ACTH may be used to localize the site of insufficiency to either anterior pituitary or the adrenal cortex (a combined lesion at both sites would not be detected). The results of ACTH administration to deficient and control animals are shown on figure 1 and table 3. Again a notable unresponsiveness of the deficient animal as indicated by both indices is evident, and it is reasonable to conclude from these data that at least one site of the insufficiency is the adrenal cortex.

Response to cortisone. There is another point which must be emphasized in the evaluation of the above data—namely, that there may exist a qualitative difference between the eosinophiles and lymphocytes of the deficient animal such as to make them refractory to a given amount of cortical hormone. Therefore, the response of these elements to exogenously administered 11-oxysteroid (cortisone) was measured. These data are presented in figure 1 and table 3. In contrast to the above results, it may be seen that the responses of the deficient and control animals are practically identical.

DISCUSSION

The above data corroborate and extend the previous findings of Dumm *et al.* (1949) and point to the existence of a degree of unresponsiveness of the pituitary-adrenal cortical mechanism of pantothenic acid-deficient

TABLE 3. THE RESPONSE OF LYMPHOCYTES OF CONTROL AND DEFICIENT ANIMALS TO EPINEPHRINE, ACTH, AND CORTISONE

Group and treatment	0 hours*	4 hours*	% change (mean)
Epinephrine:			
Control	9,080 ± 216	6,522 ± 433	−28
Deficient	11,040 ± 1,044	10,380 ± 570	− 6
ACTH:			
Control	8,978 ± 618	5,788 ± 138	−36
Deficient	8,402 ± 702	7,625 ± 630	− 9
Cortisone:			
Control	10,409 ± 1,110	5,230 ± 923†	−50
Deficient	10,380 ± 1,140	5,408 ± 711†	−48

* Data given as mean no. lymphocytes per mm.^{3} with S.E.
† Indicates statistically significant drop ($p = <0.01$).

rats. They further tend to indicate that at least one site of this malfunction is the adrenal cortex itself. This is not surprising in view of the previous anatomical studies on the adrenal of the pantothenate deficient rat mentioned above.

The mechanisms operating to produce this insufficiency are obscure. Some investigators have advanced the hypothesis that some of the pathological findings of the pantothenate-deficient animal may be on the basis of hypersecretion of the adrenal cortices in response to "alarming stimuli" (Deane and McKibbin, 1946). The evidence for this is mainly based on the findings of a hypertrophied gland and the early involution of lymphoid tissue in the pantothenic acid-deficient rat.

In mice, Melampy *et al.* (1950) have indicated a somewhat similar result. These authors report that serial lymphocyte counts in mature mice developing pantothenic acid-deficiency reveal a period of 5–6 weeks at which time the lymphocyte counts were relatively low. Coincidentally there was involution of the thymus and lymphoid tissue and a slowly developing hypertrophy of the adrenal cortex, with depletion of the lipoid material. The prolonged period of lymphopenia was then superseded by a stage of lymphocytosis. This may be taken to indicate the development of a stage of adrenal insufficiency, if the use of chronic lymphocyte counts be accepted as a valid measure of adrenal activity; this assumption, it would seem, involves some risk.

Our data indicate no sign of cortical hyperactivity. Rather, they point to a rather striking cortical deficiency, although, it is to be remembered that our animals had been on the deficient diet for 5–6 weeks before being tested. It is quite reasonable to suppose, however, that an initial period of hyperactivity may occur although the causes of this phase of the deficiency do not seem to be clear. Inanition is a known potent activator of the adrenal cortex (Dougherty and White, 1945) but as emphasized above, these animals do not develop a severe degree of inanition. Furthermore, the use of control animals restricted to the intake of the deficient animals would seem in large measure to obviate this factor in our experiments. Yet, deficient animals are reported to show a degree of thymic atrophy considerably greater than seen in pair-fed controls (McQueeney *et al.*, 1947). This implies a more specific factor, the nature of which is not known at present.

If a period of hypersecretion is involved in the pathogenesis of the lesions observed in this deficient state, then it must be followed by a period of hypofunction and presumably be important in the production of this insufficiency. Sayers (1950) in a recent review concluded that exhaustion of the adrenal cortex of a normal animal from hyperactivity has no sound basis at present. However, if the gland were deprived of a

part of the enzymatic complement necessary for synthesis of its hormone(s), it would not seem unreasonable to suppose that exhaustion could occur in meeting a normal or an increased demand for hormone(s).

It is pertinent to point out that the adrenal cortex of the rat is known to have a rather high content of Coenzyme A, a derivative of pantothenic acid (Kaplan and Lipmann, 1948), and it may be that this substance is a necessary part of the enzymatic machinery involved in the synthesis of steroid hormone(s) of the adrenal cortex. Although the exact site of action of this coenzyme in steroidal biosynthesis cannot as yet be ascertained, from biochemical studies of Conenzyme A in other tissues, it seems probable to tentatively assign it some role closely linked with the utilization of "active acetate," (Lipmann, 1945; Lipmann and Kaplan, 1946; Soodak and Lipmann, 1948; Olson *et al.*, 1949; Millican *et al.*, 1949).

SUMMARY

Eosinophile and lymphocyte counts were made in pantothenic acid-deficient (5–6 weeks) and pair-fed control rats following the administration of epinephrine, ACTH, and cortisone. The deficient animals showed insignificant changes to both epinephrine and ACTH, although the changes observed after cortisone were almost identical to those seen in the control animals. These findings suggest that at least one site of functional insufficiency of the pantothenic-deficient rat is in the adrenal cortex, although an additional lesion in the anterior pituitary cannot be ruled out. It is suggested that pantothenic acid, acting as an integral part of Coenzyme A, is important for the biosynthesis of steroid hormones by this endocrine gland, probably by acting in some metabolic processes involving the utilization of "active acetate." Further studies related to this problem are in progress and will be reported at a later date.

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ADRENAL SUCCINIC DEHYDROGENASE ACTIVITY DETERMINED BY THE REDUCTION OF TETRAZOLIUM SALT BY ADRENAL HOMOGENATE

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AN ASSESSMENT of the secretory activity of the adrenal cortex may be obtained by measuring the level of cortical hormones or their metabolites in the blood or urine. However, such methods are not readily adaptable to small animals such as the rat and it is in this animal especially that a second measure of adrenal function has been used, namely the determination of the ascorbic acid and/or cholesterol concentrations of the adrenal cortex. It has been shown that situations associated with a mobilization of cortical hormones are usually accompanied by alterations in these constituents. These indices are useful in acute experiments but are of doubtful value in chronic experiments (Sayers, 1950).

It is not unreasonable to suppose that the activity of the enzyme systems of an endocrine organ might bear a direct relation to its secretory activity. For example, it has been shown that the succinic dehydrogenase activity of the corpus luteum paralleled increasing endocrine activity of this organ (Meyer *et al.*, 1947). Recently histochemical studies have been reported suggesting that the activity of the enzyme systems of the adrenal cortex is correlated with the activity of the gland (Zweifach *et al.*, 1951).

The present study was undertaken to determine if the enzyme activity of adrenal homogenates would provide another means of studying adrenal function.

METHOD

Kun and Abood (1949) showed that triphenyl tetrazolium chloride could act as an hydrogen acceptor for succinic dehydrogenase. This substance is a colorless water soluble compound which on reduction gives rise to a highly colored water insoluble formazan, readily extracted with lipid solvents and estimated colorimetrically. Kun and Abood measured the degree of reduction of triphenyl tetrazolium salts by homogenates of kidney, liver, brain and testis tissues, considering it an index of the succinic dehydrogenase content of the tissue.

Certain modifications were made in the methods described by Kun and Abood and the following procedure was standardized on as giving reproducible results:

10–40 mgm. of fresh adrenal tissue are homogenized by the Potter technique (Potter &

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Elvehjem, 1936) in a medium containing 1 ml. of 0.2 M sodium succinate and 1 ml. of 0.1 M phosphate buffer. To the homogenate is then added successively 0.5 ml. of 0.2 M sodium succinate (pH 7.4), 0.5 ml., of phosphate buffer (pH 7.4), 0.5 ml. of 1% triphenyl tetrazolium chloride. The reaction tube is then made anaerobic by replacing the atmosphere in it with nitrogen and the preparation placed in the dark in a constant temperature bath at 37° C. for 60 minutes.

To the tube is then added exactly 5 ml. of ethyl acetate and shaken vigorously. The ethyl acetate is separated by centrifugation, a portion removed, dried with anhydrous sodium sulfate and the optical density determined on a Beckman B spectrophotometer at 485 mμ.

The values obtained are related to a standard curve prepared by completely reducing amounts of tetrazolium salt, from 20–100 μgm. in 4 ml. of phosphate buffer (pH 7.4), with a few crystals of sodium hydrosulfite, extracting the formazan produced with 5 ml. of ethyl acetate and measuring the optical density as described above. Under these conditions duplicate determinations on adrenal homogenate agreed with 3–5%.

After homogenization and before adding the reagents a suitable aliquot of the homogenate was removed for determination of total nitrogen, carried out by means of the micro-Kjeldahl procedure.

TABLE 1. ADRENAL SUCCINIC DEHYDROGENASE UNDER VARIOUS CONDITIONS
(Expressed as μgm. Tetrazolium reduced)

Treatment	No. of animals	Adrenal weight mgm.	μgm. tetrazolium per 100 mgm.*	mgm. N per 100 mgm.*	μgm. tetrazolium per mgm. N
Normal	10	30.6	252 ± 26	2.07 ± .17	122
4° C., 4 hours	5	32.9	370 ± 17	2.54 ± .38	145
4° C., 7 days	5	43.0	411 ± 51	2.53 ± .28	163
ACTH, 5 mgm. 4 hours previously	5	35.5	350 ± 20	2.55 ± .18	137
ACTH, 5 mgm. b.i.d. ×7	5	39.0	321 ± 14	2.28 ± .14	141
Cortisone, 5 mgm. daily ×7	6	25.5	158 ± 36	2.28 ± .40	69

* Mean ± s.d.

RESULTS

In Table 1 are shown the results of determinations of succinic dehydrogenase activity of the adrenals of young adult male albino rats subjected to a variety of conditions.

It will be seen that when the animals were exposed to the stress of cold (4° C.) for either four hours or one week there was an increase in the succinic dehydrogenase activity of the adrenals. Similar results were obtained four hours after a single subcutaneous injection of 5 mgm. of ACTH and also following seven days treatment with ACTH. On the other hand cortisone subcutaneously for seven days in doses sufficient to cause some involution of the gland resulted in a decreased level of enzymatic activity.

These changes in enzyme activity were apparent irrespective of whether the activity was related to the gland weight or to the nitrogen content of the gland.

DISCUSSION

The results reported here are in general agreement with those of Zweifach *et al.* (1951), who observed the deposition of formazan in the lipid of living adrenal slices taken from animals subjected to adrenal stimulation or inhibition. However these investigators found that in conditions of acute stress or short periods of treatment with ACTH were associated with defective staining of the tissues with reduced tetrazolium. This was interpreted as being possibly due to a temporary inactivity of the succinic dehydrogenase system during acute stress. However, the observations may have been due to the depletion of the adrenal lipid which also occurred; there thus being no medium for the formazan to be dissolved in.

It was thought that relating the amount of tetrazolium reduced to the adrenal nitrogen rather than to gland weight might give a more precise index of enzyme activity. In the present series similar results were obtained using both of these means of expression.

It is noteworthy that short term stimulation of the adrenal such as follows 5 mgm. of ACTH or four hours' exposure to cold lead to an appreciable increase in adrenal weight, accompanied by an increase in nitrogen content. This suggests that the formation of new protoplasmic elements or the growth of pre-existing ones is a rapid process. The adrenals of cortisone treated animals though showing a marked decline in weight and a slight fall in nitrogen content when compared to the normal glands, actually increased their nitrogen concentration. This suggests that in the involuted gland there was a relatively greater loss of non protoplasmic than of protoplasmic elements.

It is realized that in the above experiments the adrenal medulla contributes part of the nitrogen and possibly also part of the succinic dehydrogenase which were measured. However, the results suggest that the procedure can provide useful information concerning the functional activity of the cortical cells of the adrenal gland.

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THE ADRENAL CORTEX OF THE PANTOTHENIC ACID-DEFICIENT RAT: CARBOHYDRATE METABOLISM[1,2]

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INTRODUCTION

IN A previous communication from this laboratory (Winters *et al.*, 1952) preliminary evidence was presented suggesting that the pantothenate-deficient rat was in fact insufficient with respect to functions usually attributed to the C-11 oxysteroid of the adrenal cortex. This paper is concerned primarily with the disturbances in carbohydrate metabolism which these animals demonstrate.

Previous studies on this problem are somewhat confusing and thus far have failed to clearly define the presence or absence of significant metabolic abnormalities related to adrenal function. In a small number of pantothenic acid-deficient rats in which alloxan diabetes had been produced, Irwin and Ralli (1948) could discern no abnormalities peculiar to the deficient group which were not seen in the controls. However, in this experiment the number of animals made diabetic was small and the diabetes produced was inconstant. Deane and McKibbin (1946) measured the blood sugar and non-protein nitrogen (NPN) in deficient and control animals during the development of the deficiency. They observed no significant differences between the two groups, although the deficients did show a suspicious rise in the NPN at the 6th week, the longest interval studied. It is to be pointed out, however, that these animals were not in the fasting state at the time of the observations.

In pantothenic acid-deficient dogs, Schaefer *et al.* (1942) have described a dramatic syndrome, characterized by sudden prostration, coma, tachypnea, tachycardia, convulsive movements of the extremities and vomiting.

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Chemical studies on such animals revealed findings suggestive of adrenal insufficiency—namely a lowering of the blood glucose, a tendency toward hypochloremia and azotemia. These findings, however, cannot be accepted unreservedly as specific of adrenal insufficiency because of the complicating features of the clinical state of the animals (e.g. vomiting).

In order to define adequacy or insufficiency of the adrenal cortex of the pantothenic acid-deficient rat, we have studied several aspects of the carbohydrate metabolism of such animals which are known to be under control of the adrenal cortex. The results follow.

METHODS

General considerations. There are certain methodological difficulties in undertaking a study such as here reported. The measurement of adrenal function in small animals by the direct assay of the hormone in the circulating body fluids is not as yet feasible. Therefore indirect indices of adrenal cortical function must be used. There is always the possibility in applying such indirect technics to a vitamin deficient animal that the effector systems of the tissues through which the adrenal steroids exert their characteristic effect may be impaired by depletion of the vitamin.

There are several approaches which can be used to surmount this obstacle: (1) From the studies of Olson and Kaplan (1948), it is known that the adrenal cortex of the rat suffers a more severe depletion of coenzyme A (the functional form of pantothenic acid in metabolism) during the first 4–6 weeks of the deficiency than does the liver. More important, metabolic abnormalities in the liver tissue were not demonstrable at this time in the deficiency. This finding is a fortunate one for the investigator interested in measuring adrenal cortical function in such animals for it means that if he chooses an animal of such an age, he has a preparation in which the adrenal cortex is relatively depleted of coenzyme A and yet has the liver metabolically more or less intact. (2) The use of proper control experiments is of course essential. The control most needed is that which will test the metabolic integrity of the tissues directly. In most of our experiments, we have used such a control in the form of testing the response of our deficient animals to exogenously provided cortisone.

Animals. The animals and diets have been described previously (Winters *et al.*, 1952). In view of the above considerations, all experiments were carried out on animals who had subsisted for 5–6 weeks (after weaning) on the deficient diet. We would like to mention here that occasionally we have observed animals which seem to "escape" from the usual effects of the deficient diet and seem to grow normally. These animals have none of the stigmata of the deficiency. The reason for this type of behavior is not as yet clear; it may be due to increased intestinal synthesis of the vitamin. At any rate, we have avoided using such animals in our experiments simply because we do not believe they are truly deficient.

All animals for the insulin, blood sugar, muscle, and liver glycogen experiments were in the fasting state. The conditions for the phlorhizin study were identical to those of Wells and Kendall (1940a) in their studies on the phlorhizinized-adrenalectomized rat.

Drugs. Regular insulin (Lilly) was diluted appropriately with isotonic saline and was administered IP in a dose of 0.1 unit per 100 grams body weight. Adrenocorticotrophic hormone ((ACTH) a product of the Armour Laboratories) was made up in isotonic saline and administered by the routes and in the dosages indicated in the text. A saline

suspension of cortisone acetate (Merck) was injected as given in the text. Following the recommendation of McKee and Hawkins (1945), phlorhizin glycoside was made up in propylene glycol in which it is soluble with gentle heating. This material was given in a dose of 50 mg. per 100 grams body weight. The total injection volume was 0.5 ml. We have observed no untoward reactions from the use of this diluent.

Chemical. Blood glucose was determined on free flowing tail blood by the method of Nelson (1944). Urines for the phlorhizin study were collected in clean vessels and preserved with a crystal of thymol and 1 gram of citric acid. Washings from the funnels were added to each day's collection. Urine glucose was determined by the quantitative method of Benedict (1911); urine non-protein nitrogen was determined by the micro-Kjeldahl procedure after deproteinization. Liver and muscle for glycogen analysis were digested

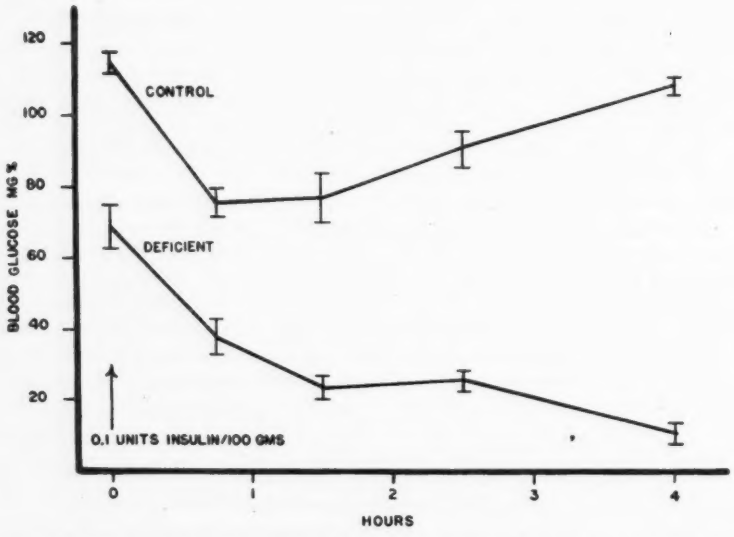

FIG. 1. The effect of insulin on the blood sugar of pantothenic acid-deficient and control rats. Curves represent mean with S. E. on 10 deficient and 5 control animals.

by the Pflüger procedure, the glucose being determined by the anthrone method (Morris, 1948; Viles and Silverman, 1949). With regard to the latter, we have found that accurately standardizing the rate of addition of the sulfuric acid and the time of heating gives more uniform results.

EXPERIMENTS AND RESULTS

Insulin sensitivity and fasting blood sugar. The adrenal cortex is one of the recognized determinants of insulin sensitivity. Previous workers had noted that the adrenalectomized animal is more sensitive to insulin than the normal animal, a state which is remedied by adrenal extract administration (Grattan and Jensen, 1940; Grattan *et al.*, 1941). The

amelioration of pancreatic diabetes by adrenalectomy is likewise evidence of the anti-insulin character of the secretion of the adrenal cortex (Long and Lukens, 1936; Long *et al.*, 1940).

As a preliminary experiment, a group of pantothenic acid-deficient (5–6 week) and pair-fed controls were given a test dose of insulin and the blood sugar measured at intervals thereafter. The results appear in figure 1, where the response of the deficient group is markedly different from that of the controls. Closer examination of these curves reveals that both are abnormal with respect to shape and time relations. The control groups show an evident degree of insulin resistance while the deficients show an increased sensitivity. With respect to the control curve, it is likely that these animals are in a state of moderate "starvation diabetes"

TABLE 1. THE FASTING BLOOD SUGAR LEVELS OF PANTOTHENIC ACID DEFICIENT AND PAIR-FED CONTROL RATS

Group	No. animals	Fasting blood sugar (mg. %)*
Control	12	112 ± 3 mg. %
Deficient	17	68 ± 7 mg. %

Data expressed as mean with S.E.
* All animals had been fasting 24 hours.
The differences are statistically significant ($p = <0.01$).

probably attendant to the reduced food intake as the result of the pair-feeding technic. This state is known to be accompanied by a degree of insulin resistance (see Peters and Van Slyke, 1946; Lundbaek, 1948). Further support for this contention stems from the observation that the control animals frequently consumed their daily allotment of food in a short time. In this respect they resemble the animals described by Tepperman *et al.* (1943), which show definite abnormalities in their patterns of carbohydrate metabolism.

However the differences between the two curves do not seem to be wholly due to these factors. That is, the deficient curve of itself is abnormal and indicative of increased sensitivity.

It was also of some interest to note that several fatalities occurred among the deficient group. It is our impression, however, that the deficient rats did not show the expected incidence of hypoglycemic convulsions in spite of the fact that the blood sugars were at such low levels; several of the deficients died during the procedure. Although we have not made a systematic study of this point, we would like to point out that coenzyme A is important in the synthesis of acetylcholine by nervous tissue (Nachmansohn and Machado, 1943; Nachmansohn and John, 1945); it is possible

that the deficient animals do not convulse as readily as the controls due to the deficit of acetylcholine synthesis.

One final point concerning figure 1 deserves comment—namely the marked disparity between the fasting blood sugars of the two groups. In table 1 we have expanded this finding with measurements from an additional group of animals. These data show a significant difference between the two groups. It will be noted that the fasting sugar levels of the control animals are somewhat higher than those usually obtained by other investigators using the same method (see e.g. Gershberg *et al.*, 1950). It should be pointed out that the animals used here were still young (5–6 weeks post-weaning) whereas most data reported on blood

TABLE 2. THE RESPONSE OF THE MUSCLE GLYCOGEN OF PANTOTHENIC ACID DEFICIENT AND CONTROL RATS TO OPERATIVE TRAUMA

Group	No. animals	Muscle glycogen (mg. %)	
		0 hours	1 hour
Control	4	660 ± 15 mg. %	508 ± 19 mg. %
Deficient	8	632 ± 10 mg. %	468 ± 9 mg. %

Data expressed as mean with S.E.
The differences are statistically significant ($p = <0.01$).

sugar levels have been on adult animals. It does not seem likely that "starvation diabetes" has any effect here, since this phenomenon is usually observed in the animal given exogenous carbohydrate. A second possibility is that the method in our hands yields somewhat higher results for some unaccountable reason.

These are some of the uncertainties of this technic in such an investigation. However as emphasized, these experiments were of an exploratory nature and were quite suggestive. One final point with regard to insulin sensitivity was also investigated. It could be argued that the deficient animals do not counter the hypoglycemic action of insulin due to a deficient secretion of epinephrine. The latter, according to some investigators (Vogt, 1947; Gershberg *et al.*, 1950), activates the pituitary-adrenal cortical axis to an increased rate of secretion.

This point is amenable to test. Deficient and control animals were anesthetized with nembutal (5 mg. per 100 grams) and after anesthesia had been achieved, the left gastrocnemius muscle was quickly removed and analyzed for glycogen. After an hour, the right muscle was similarly removed and analyzed. The results are shown in table 2. The glycogen of the muscles is known to fall in response to epinephrine (see Cori and Welch, 1942), and it is reasonable to believe that operative trauma of this nature is sufficient to elicit such a secretion. This is borne out by the

data which show clearly that the deficient animals can effect a fall in the muscle glycogen equivalent to that observed in the controls. This then is presumptive evidence that epinephrine synthesis and secretion are not materially affected by this deficiency.

Phlorhizin glycosuria. Some of the uncertainties of the insulin studies have been pointed out above. These prompted us to search for other technics for this investigation. The early work of Evans (1936) and Wells and Kendall (Wells, 1940; Wells and Kendall, 1940a and b; Wells and Chapman, 1940) clearly demonstrated marked differences in glucose and

TABLE 3. COMPARISON OF THE GLUCOSE AND NITROGEN EXCRETION OF PHLORHIZINIZED PANTOTHENIC ACID DEFICIENT AND CONTROL ANIMALS TREATED WITH ACTH AND CORTISONE

Group	No. animals	Treatment	Glucose (mg./100 gm. body wt.)	Nitrogen (mg./100 gm. body wt.)	G/N ratio
Control	20	None	410 ± 11 mg.	125 ± 6 mg.	3.3
Deficient	15	None	236 ± 7 mg.‡	74 ± 4 mg.‡	3.2
Deficient	5	2 mg. ACTH*	250 ± 7 mg.	77 ± 4 mg.	3.3
Deficient	8	4 mg. ACTH*	223 ± 13 mg.	71 ± 4 mg.	3.1
Deficient	7	2 mg. Cortisone†	362 ± 30 mg.§	116 ± 10 mg.§	3.1
Deficient	9	4 mg. Cortisone†	514 ± 24 mg.§	165 ± 10 mg.§	3.1

Data expressed as mean with S.E.
* Divided doses 12 hours apart.
† Divided doses 12 hours apart.
‡ Indicates statistical significance ($p = <0.01$) when compared to controls.
§ Indicates statistical significance ($p = <0.01$) when compared to untreated deficients.

nitrogen excretion between adrenalectomized-phlorhizinized and intact-phlorhizinized rats. Phlorhizin has the unique property of lowering the renal "threshold" for glucose (McKee and Hawkins, 1945). In the fasting state, the renal losses of glucose produce a compensatory response in the direction of increasing glucose formation from non-glucose precursors. In the adrenalectomized animal so treated, these reactions do not proceed as rapidly as in the normal animal. Exogenously administered C-11 oxysteroids will, however, restore the rate of these reactions to normal or near-normal (Wells and Kendall, 1940a; Wells, 1940).

Accordingly, a group of pantothenic acid deficient (5–6 weeks) and control animals were given phlorhizin and their glucose and nitrogen excretion measured. In addition the response to ACTH and cortisone was also assayed. The results are shown in table 3. These data show: (1) that the deficient animal is able to excrete only about 60 per cent of the glucose and nitrogen excreted by the control animal; (2) ACTH in moderate or high dosage is without effect in increasing the excretion of either substance; (3) cortisone in moderate dosage markedly increases the ex-

cretion of both glucose and nitrogen while with large doses the excretion can be raised to super-normal levels; (4) the G/N ratio remains reasonably constant in all groups.

The possibility that glucose is not excreted as efficiently by the deficient animal was also investigated. Ten deficients and 10 controls were given 500 mg. of glucose per 100 grams body weight intraperitoneally and the "extra glucose" was measured. Almost identical recoveries were observed—65 per cent in the controls and 59 per cent in the deficients. It was of some interest that several fatalities possibly of hypoglycemic causes occurred in both the untreated and the ACTH treated deficient animals. Data from these animals have not been included in the table.

Liver glycogen deposition. Earlier workers had noted that the adrenalectomized animal frequently showed very low levels of liver glycogen, a finding confirmed and extended in the classic study of Long *et al.* (1940), who showed that adrenalectomized mice and rats adequately maintained on sodium salts show a profound depletion of hepatic glycogen when fasted. With proper supplementation therapy, large increases in liver glycogen occurred, and on the basis of the increased nitrogen excretion observed under such conditions, these authors concluded that an increased rate of protein catabolism was the primary process in yielding the newly formed carbohydrate. This finding has been repeatedly confirmed and has been made the basis of numerous assay technics for adrenal corticoids. It was thus of some interest to determine the capabilities of the pantothenic acid-deficient rat in this regard.

As in the previous experiments, the 5–6 week deficient animal was used. These animals and their pair-fed controls were divided into the following groups—untreated (fasted 24 hours and sacrificed, hereafter designated 24 hours), ACTH treated animals (fasted 24 hours, given ACTH and sacrificed 12 hours later, hereafter designated 36 hours) and cortisone treated animals (fasted 24 hours, given cortisone and sacrificed 30 hours later; hereafter designated 54 hours). These time intervals were chosen following the unpublished experience of Fry, who has found them to be optimal for the detection of the responses when compared to untreated animals. Data for this experiment are presented in table 4.

Considering the untreated animals, there are slight differences between the two groups at 24 and 36 hours; these differences are not statistically significant. However, at 54 hours there is a rather well marked disparity between the two, the controls showing almost a threefold increase in liver glycogen over the deficients. In the ACTH treated groups, there is a striking increase in the liver glycogen of the controls, but little change in the level of the deficients. However, when cortisone is administered the deficient and the control groups both show a marked increase in hepatic glycogen, far exceeding the respective untreated groups.

This experiment, as well as the preceding one, show that the pantothenic acid-deficient animal has an unreactive adrenal cortex when measured by the conventional technics. That the peripheral tissues of such animals are still intrinsically capable of the response is, however, evident from the experiments using cortisone.

DISCUSSION

The results of this study confirm the previous demonstrations of the inability of the pantothenate-deficient rat to secrete adequate amounts of the adrenal steroids of the C-11oxy variety, as indicated by eosinophile and lymphocyte responses (Dumm *et al.*, 1949; Winters *et al.*, 1952)

TABLE 4. THE EFFECT OF ACTH AND CORTISONE ON PANTOTHENIC ACID DEFICIENT AND CONTROL RATS WITH RESPECT TO LIVER GLYCOGEN DEPOSITION IN THE FASTING STATE

Group	No.	Time	Treatment	Liver glycogen (mg. %)
Control	4	24 hrs.	None	94 ± 10 mg. %
Deficient	4	24 hrs.	None	79 ± 12 mg. %
Control	4	36 hrs.	None	130 ± 14 mg. %
Deficient	5	36 hrs.	None	105 ± 6 mg. %
Control	3	36 hrs.	2 mg. ACTH*	557 ± 28 mg. %‡
Deficient	4	36 hrs.	2 mg. ACTH*	127 ± 9 mg. %
Control	4	54 hrs.	None	539 ± 27 mg. %
Deficient	4	54 hrs.	None	187 ± 14 mg. %
Control	4	54 hrs.	3 mg. Cortisone†	1311 ± 91 mg. %‡
Deficient	5	54 hrs.	3 mg. Cortisone†	1088 ± 109 mg. %‡

Data expressed as mean with S.E.

* ACTH was administered in a dose of 2 mg. per 100 grams body weight subcutaneously.

† Cortisone acetate (saline suspension) was administered in a dose of 3 mg. per 100 grams body weight subcutaneously. Both drugs were given at 24 hours.

‡ Indicates statistical significance ($p = <0.01$) when compared to respective untreated group at the same time interval.

and by water intoxication studies (Gaunt *et al.*, 1946). It is to be stressed that all of these methods must be used in evaluating the adrenal cortical function of these animals in order to reduce the possibility of serious error resulting from the use of indices of adrenal function which may be invalid as the result of depletion of the vitamin from the effector systems, through which the hormone acts.

In contrast to these results abnormal sodium metabolism has not been demonstrated in the pantothenic acid deficient animal (McQueeney *et al.*, 1947), although the earlier studies of Supplee *et al.* (1942) describing an increased appetite of such animals for salt is suggestive, but not proof of abnormally large sodium loss. It will be important to establish this point before a final interpretation can be made. We are presently working on this aspect of the problem and hope to report our results in the near future.

On the basis of our present knowledge concerning the physiology of the pantothenate deficient rat, it seems likely that this vitamin plays some important role in adrenal metabolism; this hypothesis is supported by the finding of a relatively large amount of coenzyme A (the functional form of pantothenic acid) in the adrenal cortex of the rat (Kaplan and Lipmann 1948) as well as by the finding of a characteristic lesion appearing in these glands of the deficient animals (Nelson, 1939; Ashburn, 1940; Deane and McKibbin, 1946; Schultz *et al.*, 1952). There are several theoretical sites in which coenzyme A may function in this gland—either directly in the synthesis of the hormone(s) or indirectly as, for example, in the systems providing energy for these syntheses. These have been discussed in detail in the recent paper of Cowgill *et al.* (1952) and will only be commented upon here.

In other animal tissues, the common denominator of coenzyme A activity appears to revolve about the metabolism of the labile 2 carbon fragment, commonly called "active acetate." In fact, some recent evidence suggests that "active acetate" is indeed a coenzyme A-2 carbon fragment compound (see Ochoa, 1951). By analogy, coenzyme A may act in a similar fashion in the adrenal cortex, for example, in the utilization of acetate for the synthesis of cholesterol (Bloch and Rittenberg, 1942). Until recently, the adrenal cholesterol was generally regarded as being derived from the cholesterol of the circulating blood. However it now appears that the adrenal can synthesize not only cholesterol but the complete hormone from acetate (Srere *et al.*, 1948; Haines *et al.*, 1951; see Jacobsen and Pincus, 1951). It remains to be determined the quantitative importance of each of these sources of cholesterol in providing this steroid for further "differentiation" into adrenal steroid hormone(s).

A second possible site of pantothenic acid activity, which is consonant with the present knowledge of the physiology of the deficient animal, is as a component of those systems leading to the formation of the 11-oxygenated steroid, but not in those leading to the "salt" steroid. It is difficult to evaluate this suggestion at present since too little is known concerning the intimate nature of these reactions. Furthermore, authorities are not agreed as to the number and nature of the hormone(s) of this gland (see Sayers, 1950).

Finally an indirect action of this coenzyme might be in those reactions which supply energy for the syntheses and other metabolic activities of the adrenal gland. Unfortunately little is known of the nature of such processes in this tissue. It does seem likely, however, that the adrenal cortex, like other tissues, oxidizes substrates by a series of self-limited enzymatic steps in which energy is released in a step-wise fashion. Coenzyme A has recently been implicated in the reactions leading to the

synthesis of citric acid from 2 and 4 carbon precursors (Stern and Ochoa, 1951). This reaction is a key step in the tricarboxylic acid cycle and as such may assume considerable importance in the energy-yielding reactions of the adrenal cortex.

We should like to point out that the above hypotheses are only speculative. We have discussed them only because we feel that any advance in our present knowledge of the complex and intricate synthetic mechanisms of this endocrine gland is indicated. These hypotheses are based on evidence which, in the absence of more refined technics, must be regarded as suggestive *not* conclusive.

SUMMARY

1. Pantothenic acid-deficient (5–6 week) and pair-fed control rats were studied with respect to certain aspects of carbohydrate metabolism in order to gain further insight into the adrenal insufficiency which these animals demonstrate.

2. The deficient animals were found to have an increase in their sensitivity to insulin. However, the deficient animals were found to be capable of secretion of epinephrine, and the increased insulin sensitivity is probably not accountable to lack of this secretion.

3. Phlorhizinized-deficient animals fail to excrete glucose and nitrogen in amounts comparable to that observed in the controls; ACTH treatment did not materially alter the excretion of either substance, although cortisone readily restored the excretion to near-normal or super-normal levels.

4. Fasting deficient animals were compared to controls as to their ability to deposit liver glycogen in the fasting state. The deficient groups either untreated or treated with ACTH show a distinct defect in this process, which is, however, remedied with cortisone.

5. Some of the hazards and methodological difficulties of this type of investigation are discussed.

6. Several speculative hypotheses relative to the possible role of pantothenic acid in the biosynthesis of adrenal hormones are presented and discussed. It is suggested that coenzyme A, the functional form of pantothenic acid in metabolism may act in adrenal metabolism either directly in (a) the synthesis of cholesterol from acetate and/or in (b) the synthesis of the C-11 oxysteroid from cholesterol or it may act indirectly as e.g. in the reactions which energize these syntheses.

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DECREASE IN THE FORMATION OF ADRENOCORTICOTROPIN FOLLOWING ADMINISTRATION OF EPINEPHRINE

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ON THE basis of the results reported by McDermott *et al.* (1950) and Gershberg *et al.* (1950) one of the mechanisms controlling the release of adrenocorticotropin (ACTH) from the pituitary is due to an activation of the sympathetic nervous system. Through the release of epinephrine from the adrenal medulla the anterior pituitary is activated. Epinephrine is then supposed to act directly on the pituitary cells.

However Sayers *et al.* (1948) suggested that epinephrine has no direct effect on pituitary activation of ACTH but acts like other nonspecific stresses by changing the levels of cortical steroids in the circulation. They have demonstrated that the drop in adrenal ascorbic acid, which takes place one hour after the injection of epinephrine can be prevented by pretreatment with cortical steroids. Also Gelhorn *et al.* (1948) have shown that the lymphocytopenic action of epinephrine is inhibited by pretreatment with cortical steroids. Furthermore Nickerson *et al.* (1947) have reported that dibenamine, an adrenergic blocking agent, has no effect on the reduction of ascorbic acid in the adrenal following stress.

This paper presents data on the effect of epinephrine on adrenalectomized rats, i.e. animals lacking adrenal cortical steroids. Obviously in such animals the effect of epinephrine on pituitary ACTH can not be mediated through varying levels of adrenal steroids in the circulation.

METHODS AND MATERIALS

Male rats of the Long-Evans strain were used throughout this study. The animals were about 50 days of age, weighing around 200 g. The adrenalectomized rats were maintained on 1% NaCl in the drinking water. The operation was performed by the dorsal approach.

For estimation of plasma ACTH a method developed by us (Gemzell *et al.* 1951) was used. Blood from 30 rats was pooled and gave after centrifugation 70–80 cc. of plasma. For pituitary ACTH determinations the method described by Burns *et al.*

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(1949) was followed. Ten pituitaries from the group of 30 were taken and analyzed for the content of ACTH. An equivalent of 0.01 mg. of dry pituitaries was injected into each assay rat per 100 g. body weight. For the determinations of the disappearance rate of ACTH the technique described by Greenspan *et al.* (1950) was used.

As an assay method for ACTH the adrenal ascorbic acid depletion method of Sayers *et al.* (1948) was used. The assay animals were male Long-Evans rats, 40 days of age, hypophysectomized 24 hours before the assay. An Armour Laboratories ACTH preparation standardized against their La-1-A was used as a reference standard. See Gemzell *et al.* (1951).

Epinephrine hydrochloride (Adrenalin P. D. & Co. 2 mg./cc.) was used, diluted into peanut oil. A dose of 0.02 mg. epinephrine per 100 g. body weight was given subcutaneously. One injection was given every 12 hours.

RESULTS

Content of ACTH in the pituitary and the body fluids of adrenalectomized rats injected with epinephrine at the time of operation. In rats following adrenalectomy there is an immediate sharp drop in pituitary ACTH

TABLE 1. ACTH IN THE PITUITARY AND THE BODY FLUIDS OF ADRENALECTOMIZED RATS INJECTED WITH EPINEPHRINE IMMEDIATELY AFTER THE OPERATION

No. of rats	Epinephrine per 100 g. body wt.	Time of experiment	ACTH* in body fluids	ACTH* in pituitary
	mg.	hr.	μg.	μg.
30	0.02	1	—	76
30	Control	—	—	76
30	0.02	2	1.93	24
30	Control	—	10.10	44
30	0.02	6	1.70	21
30	Control	—	6.01	47
10	0.02	12	—	19
10	Control	—	—	24
30	0.02×2	24	2.02	22
30	Control	—	7.50	20

* La-1-A equivalent.

content. After 24 hours the pituitary ACTH content begins to rise and reaches a new level much above the normal. In the body fluids after adrenalectomy there is an immediate rise in the ACTH content (Gemzell *et al.* 1951).

Epinephrine administered immediately after adrenalectomy causes the pituitary ACTH content during the first 12 hours to drop still faster than after adrenalectomy alone. In the body fluids the ACTH content increases, but the increase is only a small part of the increase following adrenalectomy alone. See figure 1 and table 1.

Content of ACTH in the pituitary and the body fluids of adrenalectomized rats injected with epinephrine 6 days after the operation. Six days after adrenalectomy the content of ACTH in the pituitary and the body fluids

have reached a new high level. Administration of epinephrine reduces within one hour these high values both in the pituitary and the body fluids. See figure 1 and table 2.

Content of ACTH in the pituitary and the body fluids of normal rats injected with epinephrine. The average pituitary content of ACTH is

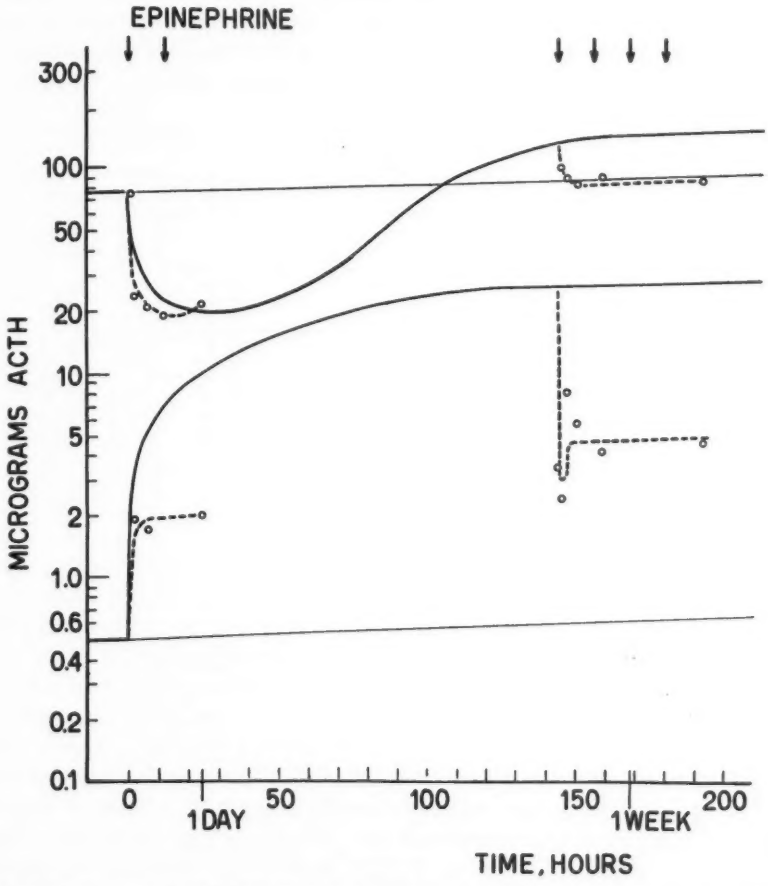

FIG. 1. Content of ACTH in the pituitary (above) and body fluids (below) of normal (thin lines) and adrenalectomized (thick lines) rats. The dotted lines represent the content of ACTH in the pituitary and body fluids of adrenalectomized rats after administration of epinephrine.

78 μg. per dry gland from a 200 g. rat. Previously by Gemzell *et al.* (1951) it has been shown that the amount of ACTH in the body fluids of a normal

200 g. rat is less than 0.50 μg. Following injections of epinephrine the ACTH content of the pituitary drops to 50 μg. in a 24 hour period. At the same time the ACTH content in the body fluids increases to 1.20 μg. i.e. more than 2 times the amount of ACTH in the body fluids of a normal uninjected rat. See figure 2 and table 3.

Determination of the ACTH space and the biological half life of ACTH in normal and adrenalectomized rats and in the same rats injected with epinephrine. The disappearance rate of ACTH injected intravenously into normal rats has been reported by Greenspan *et al.* (1950) and in

TABLE 2. ACTH IN THE PITUITARY AND THE BODY FLUIDS OF ADRENALECTOMIZED RATS INJECTED WITH EPINEPHRINE 6 DAYS AFTER THE OPERATION

No. of rats	Epinephrine per 100 g. body wt.	Time of experiment	ACTH* in body fluids	ACTH* in pituitary
	mg.	hr.	μg.	μg.
30	0.02	½	3.58	—
30	Control	—	28.90	—
30	0.02	1	2.49	104
30	Control	—	26.60	158
30	0.02	3	8.25	92
30	Control	—	29.00	162
30	0.02	6	5.90	84
30	Control	—	27.90	140
30	0.02×2	15	4.28	92
30	Control	—	28.80	174
30	0.02×2	48	4.79	87
30	Control	—	20.80	160

* La-1-A equivalent.

adrenalectomized rats by Gemzell *et al.* (1951). It is found that the biological half life of ACTH is not influenced by adrenalectomy. This study shows that administration of epinephrine does not alter the biological half life of injected ACTH. In normal rats, adrenalectomized rats and normal and adrenalectomized rats injected with epinephrine it is 4 to 10 minutes with an average value of approximately 5 minutes. After adrenalectomy the ACTH space is not significantly altered. Following administration of epinephrine however at zero time 11% of the injected ACTH is found in the total plasma compared to 6% in the total plasma of normal or adrenalectomized rats. Assuming that this concentration at zero time results from an equal dilution of ACTH and that no ACTH is destroyed or removed, the ACTH space is found to be 23% of the body mass in epinephrine injected rats compared to 43% in normal or adrenalectomized animals.

DISCUSSION

The inhibitory effect of epinephrine on pituitary ACTH in adrenalectomized rats, i.e. rats lacking cortical steroids, may be directed either to the

secretion of ACTH or to its formation. If epinephrine inhibits the secretion of ACTH the drop in the pituitary content of ACTH following adrenalectomy would occur at a slower rate than after adrenalectomy alone. Also the inhibitory effect of epinephrine in 6 day adrenalectomized rats would increase the content of ACTH in the pituitary. However, it appears from figure 1 that this is not the case. If, however, epinephrine has an inhibitory

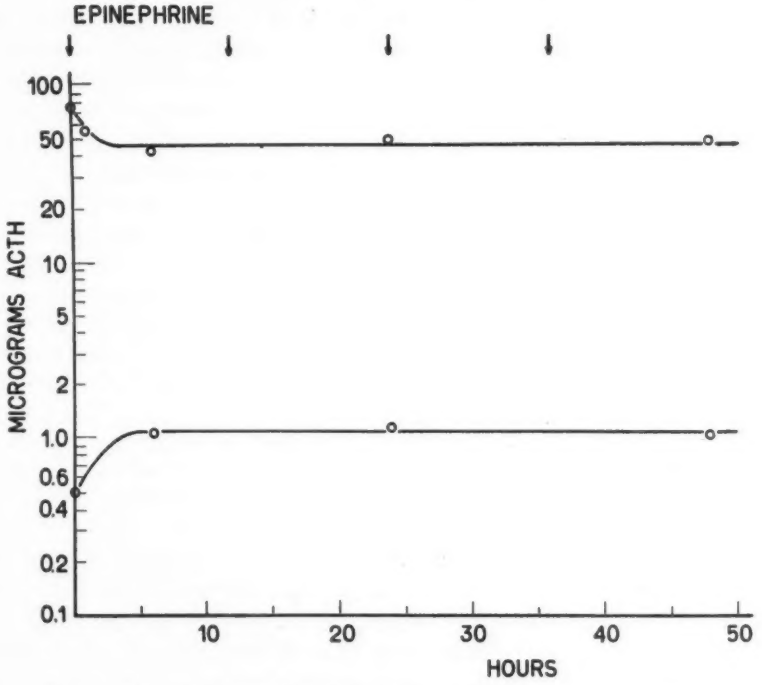

FIG. 2. Content of ACTH in the pituitary (above) and the body fluids (below) of normal rats after administration of epinephrine.

TABLE 3. ACTH IN THE PITUITARY AND THE BODY FLUIDS OF NORMAL MALE RATS INJECTED WITH EPINEPHRINE

No. of rats	Epinephrine per 100 g. body wt.	Time of experiment	ACTH* in body fluids	ACTH* in pituitary
	mg.	hr.	μg.	μg.
220	Control	—	0.50	78
10	0.02	1	—	56
30	0.02	6	1.06	42
30	0.02×2	24	1.20	50
30	0.02×4	48	1.10	48

* La-1-A equivalent.

effect on the formation of ACTH, the content of ACTH in the pituitary would decrease faster than after adrenalectomy alone. This would also result in a decreased content of pituitary ACTH when epinephrine is administered to rats with an increased ACTH formation. The data presented in figure 1 favor this second hypothesis. Thus it would appear that epinephrine acts direct on the pituitary and inhibits its formation of ACTH.

This study also shows that administration of epinephrine to normal rats increases the concentration of ACTH in the body fluids. This increased concentration may be an indication of either an unchanged discharge of ACTH from the pituitary into a smaller space or an increased secretion of ACTH from the pituitary. However the total amount of ACTH in the body fluids of a 200 g. rat injected with epinephrine is more than twice as much as the amount of ACTH in an untreated rat, even if the smaller ACTH space is taken under consideration. Since the removal rate of ACTH is identical in both groups of animals it seems clear that epinephrine has a stimulatory effect on ACTH release from the pituitary.

In normal rats during a 48-hour period following administration of epinephrine there is an increase of ACTH in the body fluids from less than 0.5 μg. to 2 μg. At the same time the ACTH content of the pituitary drops from 78 μg. to 50 μg.

However experiments with estrogen have shown that during a 48-hour period a still higher level of ACTH (5 μg.) could be found in the body fluids of rats without any drop in the pituitary content of ACTH (Gemzell, in press).

Estrogen is supposed to act through the mediation of the cortical steroids. It decreases the concentration of these steroids in the body fluids and through this mechanism brings about an increase in the ACTH secretion from the pituitary. When there is no drop in the pituitary content of ACTH, it seems reasonable to assume that the increased secretion rate of ACTH is immediately followed by a similar increase in its formation rate.

After administration of epinephrine, however, a smaller concentration of ACTH in the body fluids is accompanied by a drop in the content of ACTH in the pituitary. This drop, as has been shown after estrogen administration, is not necessary in order to maintain the increased secretion of ACTH to the body fluids.

This study has shown that epinephrine administered to normal rats, i.e. rats with cortical steroids, has a stimulatory effect on ACTH secretion and a second effect which lowers the content of ACTH in the pituitary. It seems as if this second effect inhibits the formation of ACTH in the pituitary. Thus it would appear that in normal rats epinephrine has a

direct effect on pituitary ACTH formation as has already been shown in adrenalectomized rats. Following an injection of epinephrine the increased secretion rate of ACTH is not followed by an increased formation rate.

It is suggested tentatively that the inhibitory effect of epinephrine on the formation of ACTH depends on a partial constriction of the blood vessels leading to the pituitary. In any case a similar condition has been shown to occur in other parts of the brain. Thus a decreased blood supply to the pituitary would explain the diminished formation rate of ACTH in the gland.

The dose of epinephrine used in this study may be too large and have an unphysiological effect. However, the data show that a single injection of 0.02 μg. epinephrine in oil administered subcutanously has an effect on ACTH formation lasting at least 12 hours. Assuming that the absorbtion of epinephrine occurs at the same rate throughout the whole 12 hour period and all the epinephrine is absorbed, only 0.03 μg. of epinephrine is brought into the circulation per minute. Given subcutaneously during one hour this dose of epinephrine is sufficient to produce a marked adrenal ascorbic acid depletion in a normal rat. This amount of epinephrine seems to be close to the minimal effective dose since a quantity of 0.015 μg. per minute given during one hour under identical conditions is unable to reduce the adrenal ascorbic acid in the same kind of rats.

CONCLUSION

In adrenalectomized rats, epinephrine causes the level of ACTH in the body fluids to drop through a direct inhibitory effect on the formation of ACTH in the pituitary In normal rats epinephrine causes the level of ACTH in the body fluids to increase through an indirect stimulatory effect on the ACTH secretion from the pituitary

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THE EFFECT OF GENERAL DIETARY DEFICIENCIES ON THE RESPONSE OF THE PROSTATE OF THE ALBINO RAT TO TESTOSTERONE PROPIONATE[1]

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THIS study of prostatic growth in the albino rat subjected to various types of general dietary deficiencies was undertaken to attempt to demonstrate the effect or lack of effect of ingested food on the response of the prostate of castrate rats to subcutaneous testosterone propionate.

Moore and Samuels (1931) pointed out that the atrophy of seminal vesicles and prostate which resulted from inanition was not due to a failure to respond to androgen but to a failure of androgen production. This has been confirmed by many workers. It has been shown that the injection of chorionic gonadotropin into intact starving rats results in the growth of the male accessories presumably through stimulating male hormone production (Mulinos and Pomerantz, 1941). Pazos and Huggins (1945) demonstrated the ability of completely starved dogs to secrete prostatic fluid under combined testosterone and pilocarpine stimulation and to undergo prostatic growth through testosterone administration under these conditions.

More recently, Goldsmith and Nigrelli (1950) have shown that the response of the male accessories in the castrate mouse to testosterone propionate is definitely influenced by the dietary intake. The findings of Kline and Dorfman (1951) demonstrate that the effectiveness of testosterone propionate in stimulating seminal vesicle growth is reduced in rats by moderately severe inanition.

EXPERIMENTAL

The response of 200 gm. castrated white male rats of the Wistar strain to testosterone propionate was studied in the following groups of rats: 1) those on reduced rations of stock diet; 2) those in which vitamin-free casein and tap water were the only sources

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[1] This work was done wholly under a Grant-in-Aid from The American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

of food; and 3) those in which glucose and tap water were the only sources of food. Castrated rats of the same initial weight, but given access to 20 gm. of stock diet daily, served as controls.

In the first experiment the prostatic weights resulting from testosterone propionate injections in castrate male rats on a 20 gm. stock diet[2] daily and on 5 gm.[3] stock diet daily were compared. Forty-five 200 gm. male rats of the Wistar strain were separated into these two dietary groups, 17 being placed on normal stock diet and 28 on reduced stock diet ration. Five days after the animals were placed on the restricted food intake, all rats with the exception of four in each division were castrated. Two days later, by which time the rats on decreased rations of stock diet had lost one-fifth to one-fourth of the pre-inanition weight, subcutaneous injections of testosterone propionate in oil[4] were begun. Groups of 3 to 4 normally fed and 4 to 10 inanition rats receiving 0.05, 1.0, 2.0, and 4.0 mg. of testosterone propionate per day for 20 days were studied. They were sacrificed and autopsied 3 days after the final injection.

In the second experiment albino rats of the Wistar strain were castrated and 4 weeks later, when they weighed 225 to 240 gm., they were placed on special diets. Twelve rats had access to 20 gm. of stock diet daily; 14 were maintained on water and vitamin-free casein; 17 were given vitamin-free casein containing 1½% sulfa mixture (sulfaguanidine 0.5%; sulfasuxidine 1%); 13 were maintained on vitamin-free casein supplemented by subcutaneous injections of 1 cc. of Lilly's crude liver extract once weekly. The sulfa mixture was added to the diet in an attempt to inhibit intestinal bacteria and thus eliminate from consideration the products of their metabolism. One week after the special feedings were instituted, rats, in each group were begun on injections of testosterone propionate in 0.1 mg. and 4.0 mg. doses given subcutaneously every other day for 8 injections. The rats were autopsied two days after the eighth injection.

In the third experiment 48 male albino rats were castrated. Three weeks later when they weighed about 200 gm., they were placed on special diets. Fifteen had 20 gm. of stock diet available daily; 18 were placed on glucose only; 7 received stock diet containing a 1½% sulfa mixture; and 8 received glucose containing a 1½% sulfa mixture. Two weeks after the special diets had been instituted, groups of animals were placed on subcutaneous testosterone propionate in doses of 0.1 and 4 mg. every other day. Two days after the eighth injection they were killed with ether and autopsied.

In all groups body weights were recorded once weekly and at autopsy. Ventral and posterior prostate, adrenals and pituitary weights were obtained at autopsy on a Roller-Smith balance.

RESULTS

The results for the group of inanition rats show that all animals receiving testosterone propionate demonstrated a marked androgenic response as measured by the increase in prostatic weight (Table 1). Despite

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[2] Purina Laboratory Chow.

[3] Two rats of same weight were kept in each cage and two 5 gm. pellets were placed in the cage daily. If one rat appeared to dominate the other in the struggle for available food, they were separated.

[4] The crystalline testosterone propionate was kindly supplied by Dr. Edward Henderson of the Schering Corp.

TABLE 1. RESULTS OF DECREASED INTAKE OF STOCK DIET ON RESPONSE TO VARYING DOSES OF TESTOSTERONE PROPIONATE

Adult male rats	Testosterone propionate (mg. per day)	Number of rats	Initial body wt. (grams)	Final body wt. (grams)	Change body wt. (grams)	Total prostate wt. (mg.)	Ventral prostate wt. (mg.)	Posterior prostate wt. (mg.)
Intact Gonads								
Stock Diet	—	4	206	267	+61	507 (372–580)	319	188
Inanition	—	4	216	150	−66	248 (69–339)	162	86
Castrate								
Stock Diet	0.1	4	220	279	+59	400 (312–519)	240	160
Inanition	0.1	10	216	145	−71	365 (192–516)	224	141
Stock Diet	1.0	3	198	249	+51	993 (817–1248)	625	368
Inanition	1.0	5	217	157	−60	990 (882–1104)	631	359
Stock Diet	2.0	3	212	263	+51	1105 (953–1241)	639	466
Inanition	2.0	5	211	150	−61	951 (809–1151)	638	313
Stock Diet	4.0	3	210	243	+33	1241 (1177–1321)	763	478
Inanition	4.0	4	211	156	−55	979 (845–1213)	607	372

TABLE 2. RESULTS OF DIETARY INTAKE OF VITAMIN-FREE CASEIN ON RESPONSE TO TESTOSTERONE PROPIONATE

Adult male rats	Testosterone propionate (mg. every other day)	Number of animals	Initial body wt. (grams)	Final body wt. (grams)	Change body wt. (grams)	Total prostate wt. (mg.)	Ventral prostate wt. (mg.)	Posterior prostate wt. (mg.)
Intact Gonads								
Stock Diet	—	6	275	288	+13	701 (509–942)	441	260
V.F. Casein*	—	6	287	180	−107	332 (227–503)	206	126
Castrate								
Stock Diet	—	4	247	269	+22	40 (29–49)	20	20
Stock Diet, Sulfa Drugs	—	4	277	288	+11	44 (24–70)	25	19
Stock Diet, Liver Extract	—	4	281	305	+24	54 (42–66)	22	32
V.F. Casein	—	4	246	154	−92	23 (22–24)	9	14
V.F. Casein, Sulfa Drugs	—	4	274	177	−97	38 (26–55)	21	17
V.F. Casein, Liver, Extract	—	4	250	165	−85	32 (20–50)	18	14
Stock Diet	0.1	4	223	279	+56	236 (172–356)	134	112
Stock Diet, Sulfa Drugs	0.1	5	230	276	+46	272 (186–326)	153	119
Stock Diet, Liver Extract	0.1	5	231	277	+46	269 (237–309)	159	110
V.F. Casein	0.1	4	234	171	−63	304 (258–341)	183	121
V.F. Casein, Sulfa Drugs	0.1	4	236	173	−63	309 (273–340)	191	118
V.F. Casein, Liver Extract	0.1	5	240	162	−78	268 (109–355)	168	100
Stock Diet	4.0	4	226	276	+50	885 (823–965)	528	357
Stock Diet, Sulfa Drugs	4.0	9	273	319	+46	976 (836–1126)	598	378
Stock Diet, Liver Extract	4.0	4	231	275	+44	907 (798–1062)	565	342
V.F. Casein	4.0	4	223	160	−63	704 (682–726)	437	267
V.F. Casein, Sulfa Drugs	4.0	9	281	173	−108	709 (558–863)	434	275
V.F. Casein, Liver Extract	4.0	4	231	168	−63	828 (499–1039)	544	284

* Vitamin-free Casein.

the great differences in body weight between normally fed and inanition groups, the average prostatic weights for the inanition and normally fed groups receiving 0.05 mg. and 1 mg. of testosterone propionate daily are almost identical. With increasingly large doses of testosterone there is no observable increase in the level of prostatic weight in the rats on restricted diet; the prostates of rats on stock diet continue to increase in weight. The average prostatic weights of the normally fed animals is approximately one-third larger at the 4 mgm. daily dosage level than the prostate of the partially starved animals.

In the group of rats fed vitamin-free casein a marked prostatic growth

TABLE 3. RESULTS OF DIETARY INTAKE OF ONLY GLUCOSE ON RESPONSE TO TESTOSTERONE PROPIONATE

Adult male rats	Testosterone propionate (mg. every other day)	Number of animals	Initial body wt. (grams)	Final body wt. (grams)	Total prostate wt. (mg.)	Ventral prostate wt. (mg.)	Posterior prostate wt. (mg.)	Final body length (inches)
Intact								
Stock Diet	—	5	223	306	582 (349–868)	377	205	8.9
Glucose	—	5	212	127	153 (60–279)	102	51	7.8
Castrate								
Stock Diet	—	5	205	303	19 (12–30)	12	7	8.8
Glucose	—	4	197	118	26 (14–43)	15	11	7.6
Stock Diet Sulfa	—	3	207	320	13 (10–18)	6	7	8.7
Glucose Sulfa	—	3	192	112	18 (15–22)	10	8	7.4
Stock Diet	0.1	5	206	283	194 (152–241)	113	80	8.7
Glucose	0.1	7	206	125	202 (98–294)	124	78	7.5
Stock Diet Sulfa	0.1	4	216	355	224 (172–309)	128	96	9.1
Glucose Sulfa	0.1	5	196	117	185 (140–239)	107	77	7.5
Stock Diet	4.0	5	212	300	797 (669–922)	512	285	8.8
Glucose	4.0	7	195	111	425 (218–605)	283	142	7.5

is once again seen in all animals receiving testosterone propionate (Table 2). Again, despite the marked weight loss, the response of the prostate in all groups at the 0.05 mg. daily level was in the same range. In the 2 mg. per day (4 mg. every other day in 1 cc. sesame oil) groups, the castrated rats on stock diet showed a greater prostatic weight than any of the groups on vitamin-free casein. At this high dosage level, the animals receiving a weekly injection of 1 cc. of crude liver extract showed slightly greater prostatic growth than those on vitamin-free casein and vitamin-free casein containing the sulfa drugs. The addition of sulfa drugs and the use of subcutaneous crude liver extract had only a slight effect on the animals on normal diet. Furthermore, the probable decrease in number and activity of intestinal bacteria resulting from the use of the sulfa drugs did not result in any significantly altered response by the prostates of animals on those drugs and vitamin-free casein.

In the group of rats receiving only glucose (Table 3), results were obtained similar to those in the vitamin-free casein group. At the 2 mg. daily dosage level, the total prostatic weight was almost twice as large in the normally fed as in the glucose fed castrates. Dry weight determination showed the water content of the prostates of glucose fed rats to be slightly greater than that of the normally fed ones.

In all groups of intact rats subjected to various procedures resulting in a weight loss, a decrease in the weight of the male accessories can be seen.

DISCUSSION

The rate of prostatic growth of normally fed castrate rats injected with testosterone propionate exceeds that of the castrate rats on deficiency diets only at the higher dosage levels of testosterone propionate. The most striking weight differences were noted in the comparison of the normally fed and glucose fed castrates on 2 mg. of testosterone propionate daily (Table 3). The differences in prostatic weight between the normally fed rats and those on either reduced stock rations or vitamin-free casein were not as marked. However, it does seem that significant body weight loss from deficient dietary intake is associated with a decreased capacity for response to large testosterone propionate dosages. It seems unlikely that this decreased responsiveness is due to failure of absorption since no evidence of this was found at autopsy.

In our opinion, the lessened capacity to respond to androgen manifested by rats on deficiency diets truly represents a decrease in prostatic growth capacity under these circumstances. This decreased growth capacity of the prostate may simply be the result of a relative lack of the general factors necessary for the growth of any tissue; it may be a manifestation of the relative absence from the diet of one or more specific substances necessary for prostatic growth; or it may be that inanition has affected primarily some of the remaining endocrines, such as the pituitary, and that the decrease in their activity results in a decreased response of the prostate to testosterone propionate. We (Bunce, Harris *et al.*) have recently observed a decreased responsiveness to androgen in hypophysectomized castrate rats as compared to castrate controls. Since the hypophysectomized animals also lost weight, the reason for decreased androgenic response remains obscure. However, we must consider the possibility that the diminished pituitary activity which accompanies states of inanition may affect the response of the prostate to testosterone propionate.

Since the demonstration by Hertz (1945) that folic acid plays an important and specific role in the action of estrogen on its and organs the possibility that there may be a similar substance affecting the action

of androgen on the prostate is most intriguing. The work of Brendler (1949), Kline and Dorfman (1951) and others seems to indicate that folic acid does not have a specific role in prostatic response to androgen. The demonstration of a significant difference in prostatic growth, especially between castrate rats on normal diets and those on an intake of only glucose when treated with large doses of testosterone propionate, provides us with a useful tool in studying the effects of specific dietary substances on prostatic growth in the presence of known amounts of testosterone propionate.

SUMMARY

In an effort to determine the effect of diet on the response of the castrate rat's prostate to testosterone propionate, groups of rats were placed on dietary deficiencies of the following general types: 1) reduced intake of adequate stock diet; 2) limitation of diet to an intake of vitamin-free casein and tap water only; and 3) limitation of dietary intake to glucose and tap water. Castrate male rats fed 20 gm. of Purina Laboratory Chow daily served as controls. Rats in each of these groups were placed on varying amounts of testosterone propionate in sesame oil daily. All rats showed prostatic growth with administration of testosterone propionate. In the dosage range of 0.05 mg. daily there was little difference between the response in normally fed rats and those on deficient diets. At the 2 mg. daily dosage level there was uniformly a decreased prostatic growth in rats on reduced dietary intake. This was most pronounced when castrate rats fed only glucose were compared to those fed normal stock rations.

These observations confirm and extend those of others. It is suggested that the demonstrable effect of dietary intake on the response of the prostate of the castrate rat to testosterone propionate may be due either to a lack of general or specific dietary substances. The possibility that it may be the result of alterations in the activity of remaining endocrine organs must also be considered.

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THE GUINEA PIG AS A LABORATORY ANIMAL FOR CORTICOID EXCRETION STUDIES: MODIFICATION OF THE FORMALDEHYDOGENIC METHOD[1]

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THE object of this report is to present observations to show that the guinea pig is a sensitive and suitable animal for the study of urinary corticoids and adrenocortical activity. Very little work has been done on the determination of urinary corticoids of laboratory animals by chemical methods.

Most of the previous reports have been concerned with the excretion of 17 Ketosteroids in rabbits (De Koning *et al.*, 1948; Davis *et al.*, 1949; Kimeldorf, 1948), rats (Limpton and Miller, 1941) and mice (Karnofsky *et al.*, 1944). The determination of corticoids in urine of monkeys has been accomplished by biological assay which is a laborious and complicated method (Dorfman *et al.*, 1944). Heard *et al.* (1946) determined excretion of corticoids by the phosphomolybdic acid method in dogs. Urine of dogs, however, contains vitamin A, (Lawrie *et al.*, 1941) which reduces phosphomolybdic acid (Burstein, 1951) and thus interferes with the corticoid determination by this method.

METHODS

a) *Urine collection.* The animals were kept in metabolic cages made of galvanized wire and mounted on glass funnels 25 cm. in diameter. A net was placed 5 cm. below the bottom of the cage to filter feces. The animals were fasted for 17 hours, the period of urine collection, and fed ad libitum during the remaining 7 hours. On this regime the guinea pigs remained in excellent condition and did not lose weight for as long as 3 months. Approximately one hour after fasting was begun the nets were cleaned of feces. Usually there were no further fecal deposits during the remaining 16 hours. One cc. of chloroform was added to the collecting receptacles as an antiseptic.

b) *Corticoid determination.* The quantity of corticoids excreted in the urine of guinea pigs was too small for determination by the Daughaday (1948) formaldehydogenic method. This method was slightly modified therefore and made more sensitive. (With the present method as little as 15 μg. of Doc could be determined).

17 hour urine collections were used.

The urine was acidified to pH 1 and immediately extracted 3 times with equal vol-

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[1] This work was aided in part by a grant from the Max London Foundation.

umes of a 4:1 mixture of ether:chloroform. The ether-chloroform extract was extracted 5 times with cold n/10 NaOH, 5 times with distilled water, dried over Na_2SO_4 anhydrous and finally evaporated in vacuo at a temperature not exceeding 50° C. The residue was dissolved in glacial acetic acid and aliquots were taken for determination.

1) *The phosphomolybdic acid reduction method.* Phosphomolybdic acid was prepared according to Folin & Wu (1923) omitting, however, the tungstic acid. It has been shown by Burstein (1951) that phosphomolybdic acid oxidizes the unsaturated cyclic ketonic grouping to a much lesser extent that the phosphotungstomolybdic acid, thus permitting the more selective determination of ketol substances. Usually there was no turbidity in the extract of guinea pig urine.

2) *The Formaldehydogenic method.* 1 cc. of periodic acid reagent (as described by Daughaday *et al.*, 1948) was added to the glacial acetic acid extract and oxidation was carried out in the distillation flask. After 45 minutes, 0.5 cc. of stannous chloride solution was added followed by the addition of 0.3 cc. 12N H_2SO_4 and 2 cc. of distilled water. The formaldehyde was distilled by heating the solution with a small flame and bubbling inert gas through it. The tip of the condenser was placed beneath the surface of 3 cc. of a solution of chromotropic acid in sulfuric acid. (75 mg. of chromotropic acid was dissolved in 5.6 cc. water and cooled in ice water; 17.6 cc. of concentrated analytical sulfuric acid was added slowly and mixed.) According to Bricker & Vail (1950) chromotropic acid absorbs formaldehyde quantitatively. By distilling directly into chromotropic acid solution a greater sensitivity could be obtained than by distillation of the formaldehyde into bisulfite solution, as was done by Daughaday *et al.* The formaldehyde was distilled until 2 cc. of distillate had passed over. The condenser was then washed with 1 cc. of solution containing 3 parts of the solution used for absorption and 1 part of water. The resulting solution in the collecting centrifuge tube was 9M H_2SO_4 and was optimal in concentration for the formaldehyde—chromotopic acid reaction according to Mac Fadyen (1945). Color was developed by heating in a glycerine bath at 130° C. for 20 minutes and the volume adjusted to 6 cc. Desoxycorticosterone was used as a standard.

The crude chromotropic acid was purified as follows:

5 grams of the crude chromotropic acid were refluxed with 5 cc. of 96% ethyl alcohol. About 12 cc. of water were added until the substance dissolved. 0.5 g. Norite was then added, boiled for 5 minutes and filtered through a hot filter. About 40 cc. of acetone were added cautiously to the boiling filtered solution until all the chromotropic acid was precipitated as white flakes.

OBSERVATIONS

1) *Urinary corticoids in the male guinea pig*

The urinary corticoids in the male guinea pig were fairly constant in quantity and averaged approximately 140 μg. on the 17 hour urine collection by the phosphomolybdic acid method and 45 μg. by the formaldehydogenic method. These values were obtained only if the animals were fed uncolored vegetables such as cucumbers and white cabbage and only if the diet was constant in quantity.

On a diet of carrots the quantity of corticoids as determined by the phosphomolybdic method increased 100% or more; by the formaldehydogenic procedure the increase was much less. The cause of this increase in

phosphomolybdic acid reducing substances remained obscure. It was shown elsewhere (Burstein, 1951) that compounds with many conjugated double bonds reduced phosphomolybdic acid. The conjugated carotenoids of carrots are such compounds. However, neither vitamin A nor carotene was found in the urine of guinea pigs given carrots when it was tested by the method of Carr and Price, nor did the administration per os of vitamin A or carotene increase the phosphomolybdic acid reducing substances.

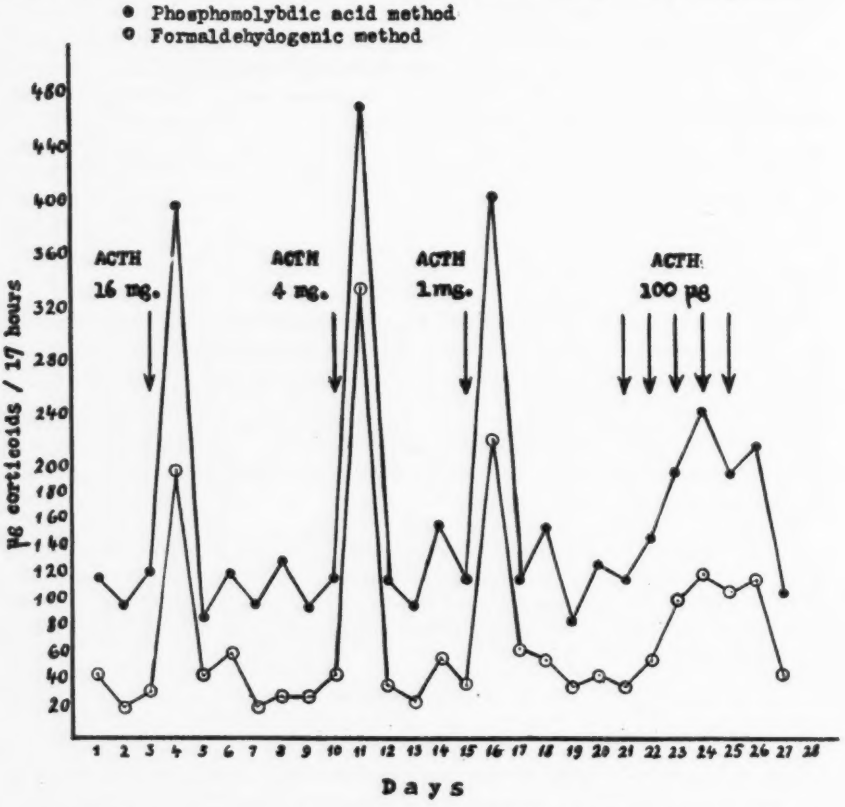

FIG. 1. Corticoid excretion pattern in the male guinea pig administered ACTH.

2) *Urinary corticoids following the administration of ACTH*[2]

The injection of ACTH in saline subcutaneously caused an immediate rise in the excretion level of corticoids as determined by both methods.

[2] ACTH was generously supplied by Dr. H. de Jager, of N. V. Oragon, OSS, Holland. This ACTH contains 1.5×the activity of the "Armour" Standard.

Administration of 100 μg. of ACTH was followed by an increase in the corticoid excretion of approximately 100%. Injection of 1 mg. was followed by a 400% increase above the baseline excretion level. Administration of 4 mg. or of 16 mg. ACTH resulted in an elevation of urinary corticoids which did not exceed that obtained with 1 mg. (Fig. 1).

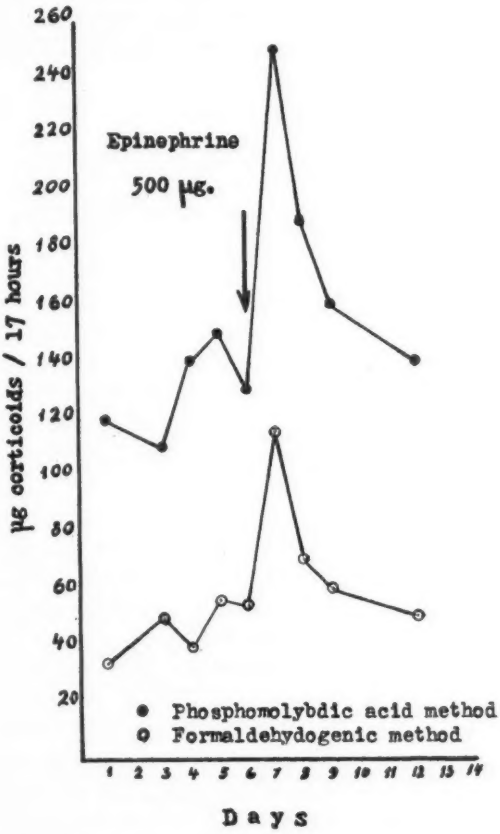

FIG. 2. Corticoid excretion pattern in the male guinea pig administered epinephrine.

3) *Urinary Corticoids following the administration of epinephrine.*[3]

The intramuscular administration of 0.5 mg. epinephrine bitartrate (dissolved in 0.5 ml. of saline immediately before use), to male guinea

[3] We are very much indebted to Dr. M. L. Tainter of Withrop-Stern Inc., New York, who generously supplied the L-epinephrine bitartrate and the D,L-Arternd.

pigs was followed by an increase in the urinary corticoids. The average elevation was approximately 100% above the baseline level. Injection of 1 mg. was not followed by a greater augmentation of urinary corticoids (Fig. 2). On the other hand a quantity of 0.2 mg. was without effect on excretion levels.

The administration of 1 mg. of norepinephine (dissolved in 1 cc. of saline immediately before use) caused a slight increase over baseline excretion levels, which was much less than that obtained by comparable doses of epinephrine.

DISCUSSION

The observations of this report show the guinea pig to be a reliable, sensitive and suitable laboratory animal for the study of urinary corticoids and adrenocortical function. All colored vegetables must be eliminated from the diet or inaccurate and unduly high results will be found.

Compared with the guinea pig the rat is a much more difficult animal to use for the investigation of urinary corticoids. Ether-chloroform extracts of rats' urine were usually turbid and prevented the employment of spectophotometric determination by the phosphomolybdic acid reagent. The color developed by chomotropic acid is sometimes non-specific (the maximum of absorption being shifted to 520 mμ instead of the formaldehyde-chromotropic color maximum which is at 570 mμ (Burstein, unpublished observations).

On the basis of body weight in comparison with the human adult male a guinea pig excretes about 10 times as much corticoids. As determined by the phosphomolybdic acid method a guinea pig of 700 grams excretes in 24 hours about 140 μg. The human male excretes about 1.6 mg. per 24 hours. It is possible that these results may be correlated with the fact that the guinea pig has the largest adrenal weight/body weight ratio among the mammals, the ratio being approximately 0.1% for the guinea pig and 0.01% for the adult human (Hartman and Brownell, 1949). On an adrenal weight basis man and guinea pig excrete about the same amount of corticoids.

The guinea pig compared to man is rather sensitive to ACTH stimulation, a dose of 100 μg. giving rise to an increase of approximately 100%. In man this dose corresponds to 10 mg. and has been shown generally not to affect corticoid excretion. It has been definitely established by McDermott *et al.* (1950) (using the eosinopenia test) that epinephrine stimulates the pituitary to secrete ACTH directly. The amounts used by these authors were very small and could not affect the metabolic activities of the organism. This led to the conclusion that epinephrine may be a specific stimulating agent. So far, a direct demonstration in man and

animals has not been reported that epinephrine augments the urinary excretion of corticoids. In the present work it has been shown that epinephrine gives rise to an increased corticoid excretion level but only if used in amounts more than 0.5 mg. per guinea pig, which exceeds physiological doses. On a comparable weight basis man would require 50 mg. in order to show an increase. This calculated human dose is in accordance with the results of Recant *et al.*, 1950, who found no substantial increase of the formaldehydogenic steroids by administering intravenously 12 mg. of epinephrine. Bliss *et al.* (1950) found in man that with a total dose of 8 mg. intramuscularly in oil the 17 ketosteroids rose by about 40% above the normal but smaller amounts of epinephrine did not affect the excretion level. Epinephrine, although it gives rise to eosinepenia in small amounts, does not augment the uric acid—creatinine ratio in the same way as ACTH does, therefore Bliss *et al.* suggested a "partial activation of the adrenal cortex by epinephrine.

Recently, Buehler *et al.* (1949) have shown that the quantity of corticoids in the urine of man is much greater when the urine is hydrolyzed by β-glucuronidase. These results, however, do not invalidate the present observations which merely record the relative quantities of corticoids excreted and which permit the study of adrenocortical function under a variety of experimental conditions.

SUMMARY

The formaldehydogenic method for the determination of urinary corticoids was modified and made more sensitive. With this method and the physphomolybdic acid method, the guinea pig was found to be a suitable laboratory animal for the study of corticoid excretion provided his diet was free of colored vegetables. ACTH and epinephrine increased within 24 hours the corticoid excretion.

Acknowledgment

I wish to express my appreciation to Prof. Bernhard Zondek for his advice and encouragement during this investigation.

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THE RELATIONSHIP OF CORTICOID EXCRETION TO OVARIAN HORMONES IN THE GUINEA PIG[1]

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THE present investigation was undertaken to study the excretion of adrenocortical hormones in the guinea pig during its estrous cycle, and to find out whether a correlation exists between ovarian function and the quantity of the excreted corticoids. It has been shown in this laboratory that the guinea pig under certain conditions is a very sensitive and suitable animal for the study of adrenocortical function by the chemical determination of urinary corticoids (Burstein, 1951).

In the guinea pig, Kolmer (1912) demonstrated that the histology of the adrenal cortex is changed during the estrous cycle, and Hunt (1940) showed that the zona fasciculata contains the greatest number of mitoses during the mid-leucocyte stage of the cycle, while the zona glomerulosa exhibits the greatest number of mitoses during the cornified cell stage. In a variety of other species histological changes of the adrenal cortex have been noted during various periods of the sexual cycle. Stilling (1898), Riddle (1923), Foster (1934), Moore *et al.* (1934), Baker (1938), Kayser & Aron (1938), Andersen & Kennedy (1932) and Mutow (1937) have described hypertrophy of the cortex in the frog, pigeon, ground squirrel, dog, hamster and rat respectively during estrus or during the breeding season. Nahm & McKenzie (1937) reported an increase in the number of dark cells and in the amount of lipid in the cortex during estrus in the sheep.

The administration of estrogens and androgens to rats has also been shown to affect the histology and cytochemistry of the adrenal cortex (Zuckerman, Bourne and Lewis (1938); Greep and Jones (1950); Allen and Bern (1942); Kimeldorf and Sodervall (1947)).

Koets (1949) demonstrated a variation in the excretion of 17-ketosteroids in women with idiopathic hirsutism, a maximum excretion being obtained at the time of ovulation. Davis and Hulit (1949) reported a distinct eosinopenia in women at ovulation time of the cycle, whereas Venning and Kazmin (1946) were unable to find any cyclical variation in the excretion of 11-oxysteroids and 17-ketosteroids in normal women.

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[1] This work was aided in part by a grant from the Max London Foundation.

METHODS

For the present investigation we have studied the excretion of urinary corticoids under the following experimental conditions: 1) in the intact female guinea pig; 2) in the spayed female and normal male pig; 3) in the spayed female administered estrogens; 4) in the spayed guinea pig administered ACTH. All animals weighed approximately 600 to 700 gms.

Urinary corticoids were determined by the phosphomolybdic acid (Heard and Sobel (1946), and formaldehydogenic methods (Daughaday *et al.* (1948) as modified by Burstein (1951). The values obtained by these two methods (in terms of desoxycorticosterone) are not the same; the values obtained by the phosphomolybdic acid method are always higher than those obtained by the formaldehydogenic method. Although no constant correlation factor between these methods could be found, nevertheless the excretion curves as determined by these two methods are generally parallel.

That the phyophomolybdic acid method generally yields higher results in human urine has been shown in the comparative investigation of Romanoff *et al.*, 1949. The cause of this higher value is not clear and is probably due to non-specific substances determined by the phosphomolybdic acid method.

The corticoids were extracted at pH 1, which is the optimal pH of extraction for urine of guinea pigs. Although by this method only a small amount of the excreted, corticoids are extracted as compared to p-glucuronidase hydrolysis (Buehler, 1950) nevertheless it can be used as a relative index of adrenocortical function.

Vaginal smears were taken twice daily: before the animals were placed in metabolic cages and after taking them out. The guinea pigs were kept in their cages for 17 hours without food, and were fed ad libitum during the remaining 7 hours. The diet was constant and consisted of fine bran and colorless vegetables such as white cabbage, cucumbers and others with a high water content. On this regimen the animals were in good condition.

RESULTS

The quantity of corticoids excreted is presented in micrograms (desoxycorticosterone) per 17 hours, the time spent in the metabolic cage. Vaginal smears are indicated on the abscissa as follows: empty spaces = negative smears showing predominantly numerous leucocytes; Ep. = appearance of epithelial cells; arrow = vaginal cornification.

I. *Corticoid Excretion Pattern in the Intact Female Guinea Pig.* (Figs. 1, 2)

The study of the excretion of corticoids in normal female guinea pigs revealed that the level of hormones in the urine changes markedly during the estrous cycle, with distinct peaks of excretion at estrus time. Guinea pig I (fig. 1) was studied during a period of 35 days. At the 4 estrous periods there are four distinct peaks of corticoid excretion, while during diestrus there is great variation in excretion at a much lower level. The value as determined by the phosphomolybdic acid method rose approximately from 120 μg./17 hours at diestrus to 220 μg./17 hours at estrus, i.e. an increase in the excretion level of 80% occurred at estrus. By the formaldehydogenic method the amount of excreted corticoids at diestrus

(approx. 60 μg.) rose to a value of 120 μg., i.e. an increase of 100% at estrus.

Similar results were obtained with another normal female guinea pig.

A third animal did not show any vaginal cornification during a period of 32 days. The animal excreted peak quantities of cordicoids at the time when epithelial cells were found in smears (fig. 2). Similarly, detailed

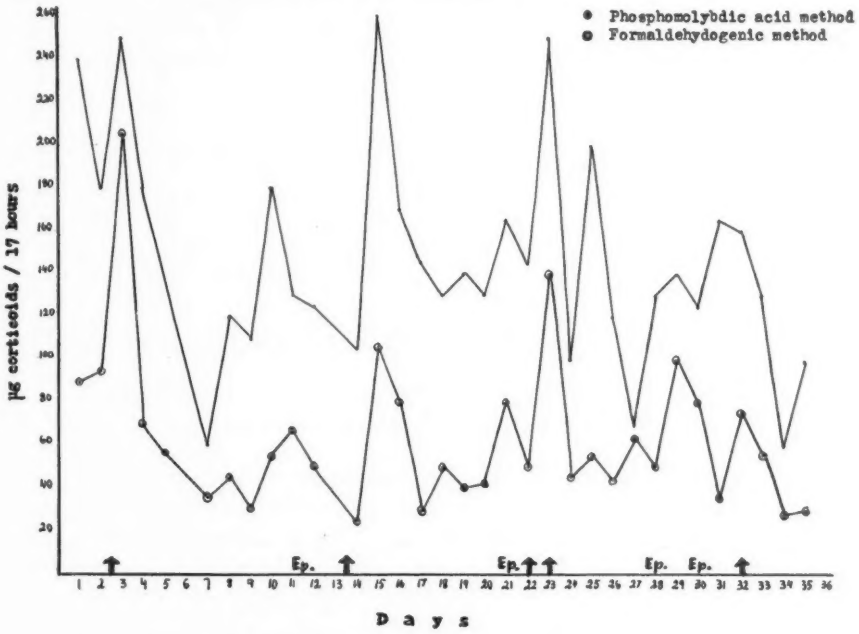

FIG. 1. Corticoid excretion pattern in female guinea pig I.

inspection of fig. 1 reveals occasional secondary peaks which occur simultaneously with the appearance of epithelial cells in the vaginal smears just before estrus.

II. *Corticoid Excretion Pattern in the Spayed Guinea Pig and in the Male Guinea Pig.* (Figs. 3, 4)

In order to determine whether the ovary is responsible for the cyclical peak corticoid excretion, an animal which showed the typical changes during estrus was spayed. Corticoid determinations were started two weeks after spaying to eliminate the factor of operative stress.

During the period of 32 days the spayed female guinea pig did not show any peaks of corticoid excretion and the vaginal smears were always

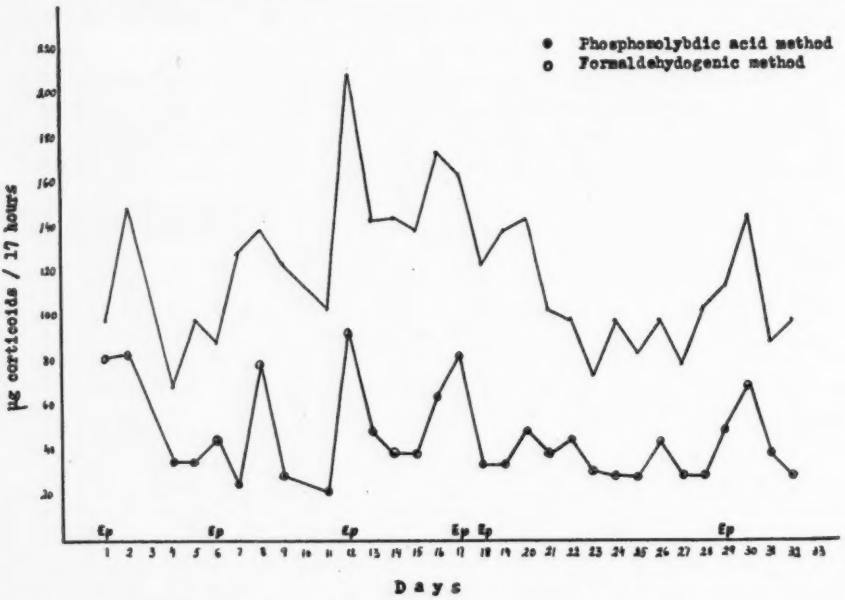

FIG. 2. Corticoid excretion pattern in female guinea pig II.

negative (fig. 3). The level of excretion was slightly lower than that found during diestrus. The excretion of corticoids was also studied in a normal male guinea pig during a period of 41 days. No peaks were apparent and the excretion level was approximately the same as that of the spayed guinea pig (fig. 4).

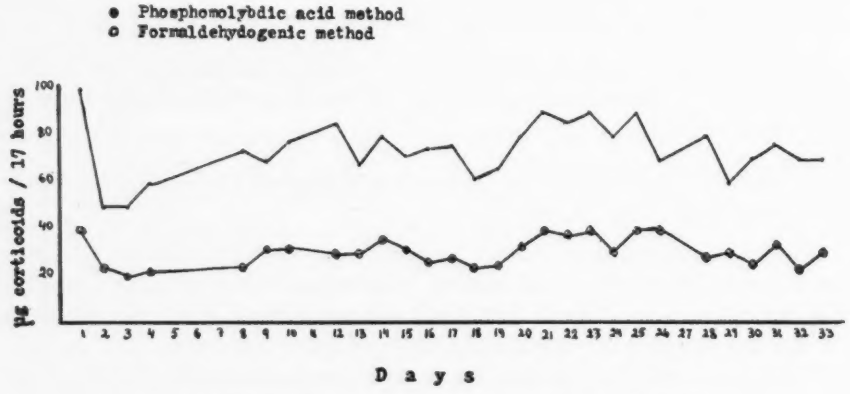

FIG. 3. Corticoid excretion pattern in the spayed guinea pig.

III. *Corticoid Excretion Pattern in the Spayed Guinea Pig Administered Estrogens and Progesterone.* (Fig. 5)

a) *Estrone*[2]

Estrone was administered subcutaneously in oil to the spayed guinea pig described above. In preliminary experiments it was shown that in the guinea pig 7 μg. of estrone are needed to produce proestrus and 100 μg. for full cornification. The administration of estrone in doses below 7 μg. had no influence upon the corticoid excretion level. 7 μg. of this reagent caused

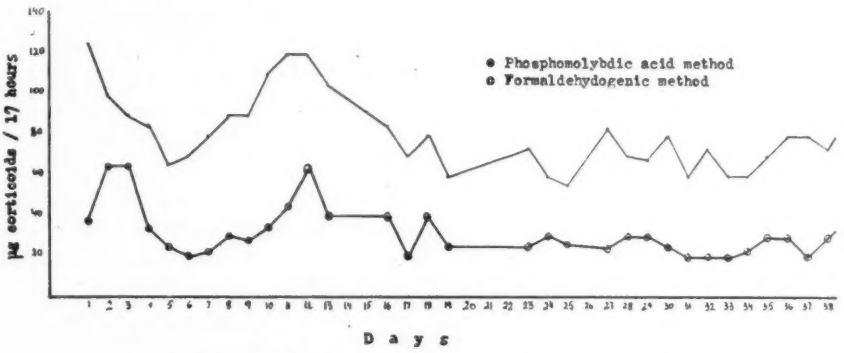

FIG. 4. Corticoid excretion pattern in the normal male guinea pig.

an immediate elevation of urinary corticoids. The increase ranged between 50–75% more than the baseline level by the phosphomolybdic acid method and between 10°–120% more by the formaldehydogenic method. Elevations of corticoid excretion of the same degree were observed after the administration of 100 μg. and 200 μg. of estrone. It is interesting to note that a 30 fold increase of dosage (200 μg.) produced only a slightly greater excretion of corticoids than was obtained by the use of 7 μg. This signifies that the increase of urinary corticoids does not correspond to the dose of administered estrogen.

b) *Stilbestrol*[3] (Fig. 6)

Similar increases in the corticoid excretion level resulted after the administration of 500 μg. of Stilbestrol, which is a relatively large dose (fig. 6). The corticoid excretion level rose with the phosphomolybdic acid method by 80–100% and with the formaldehydogenic method by 100–

[2] The estrone and progesterone were obtained from Roche-Organon Inc. U.S.A.
[3] Stilbestrol was obtained from S. B. Pernick & Co. U.S.A.

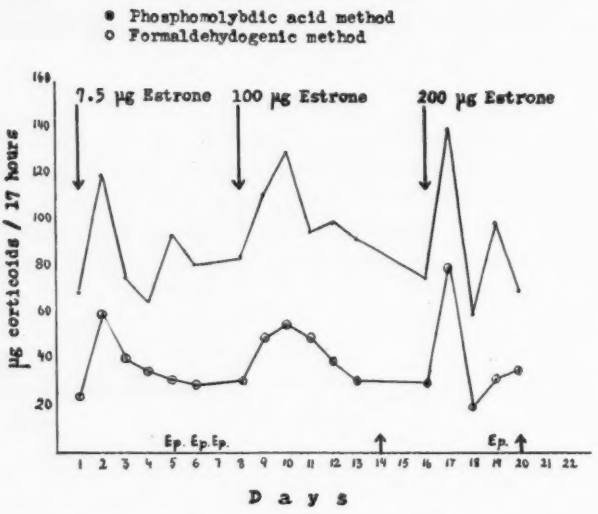

FIG. 5. Corticoid excretion pattern in the spayed guinea pig administered estrone.

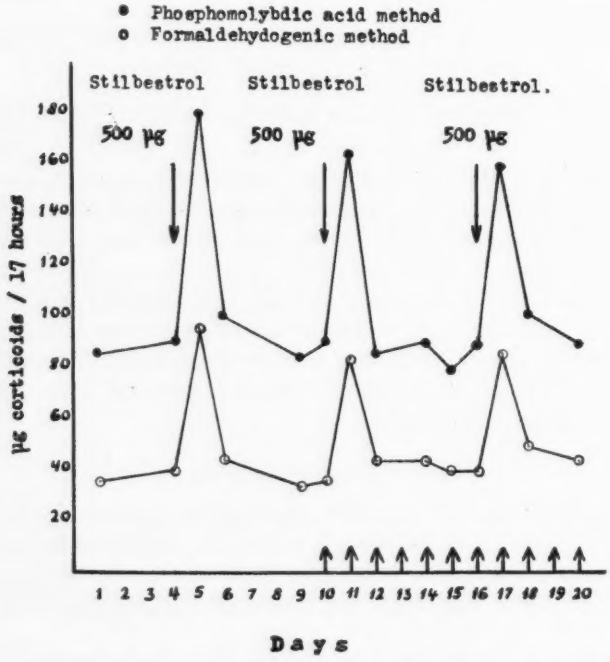

FIG. 6. Corticoid excretion pattern in the spayed guinea pig administered stilbestrol.

120%. This suggests that Stilbestrol acts like estrone and that even higher quantities of estrogenic substances do not raise the corticoid excretion level more than 100–120% above the baseline.

c) *Progesterone*[2]

Following the administration of 10 mg. progesterone to the spayed guinea pig there was no change in excretion level of the corticoids.

IV. *Corticoid Excretion Pattern in the Spayed Guinea Pig Administered ACTH.*[4] (Fig. 7)

The experiments with estrogenic hormones showed that the level of the excreted corticoids is not elevated appreciably when larger amounts of estrogens were administered. In order to see whether the adrenals were the limiting factor in this behavior, ACTH was administered to the spayed guinea pig.

Following the injection of 50 μg. of ACTH the excretion level rose approximately 100% by both chemical methods; using 100 μg. of ACTH the increase was approximately 140%. 1 mg. of ACTH caused an increase of approximately 400% (fig. 7). These results clearly show that ACTH is able to augment the excretion level of the corticoids much more than the estrogens.

DISCUSSION

The observations of the present investigation reveal an intimate relationship between estrus, estrogens and the excretion of corticoids. If the assumption is valid that urinary corticoids as determined by the present methods reflect adrenocortical function, our data show that: 1) adrenocortical function is cyclical and increased at the time of estrus, 2) adrenocortical activity is low and acyclical in the absence of the ovary and in the male guinea pig; 3) adrenocortical activity is increased by the administration of estrogens. The corticoid excretion patterns of the normal cycling female guinea pig and of the spayed animal administered estrone are similar. There is, however, a difference. An increase in corticoid excretion level is found during the guinea pig cycle at the stage of proestrus or estrus, when epithelial and cornified cells are found in the vaginal smears, i.e. at ovulation time. On the other hand in the spayed animal the corticoid level rises almost immediately, i.e. 24–48 hours following the parenteral administration of estrogens. At this time no epithelial cells or vaginal cornification are yet apparent, since they appear only several days later.

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[4] ACTH was generously supplied by Dr. H. deJager, of N. V. Organon, Oss, Holland. This ACTH contains 1.5 × the activity of the "Armour" Standard.

According to these observations we conclude that the estrogenic hormones elevate corticoid urinary levels. It is assumed that the maximal estrogen level in the guinea pig cycle occurs at ovulation time. As has been

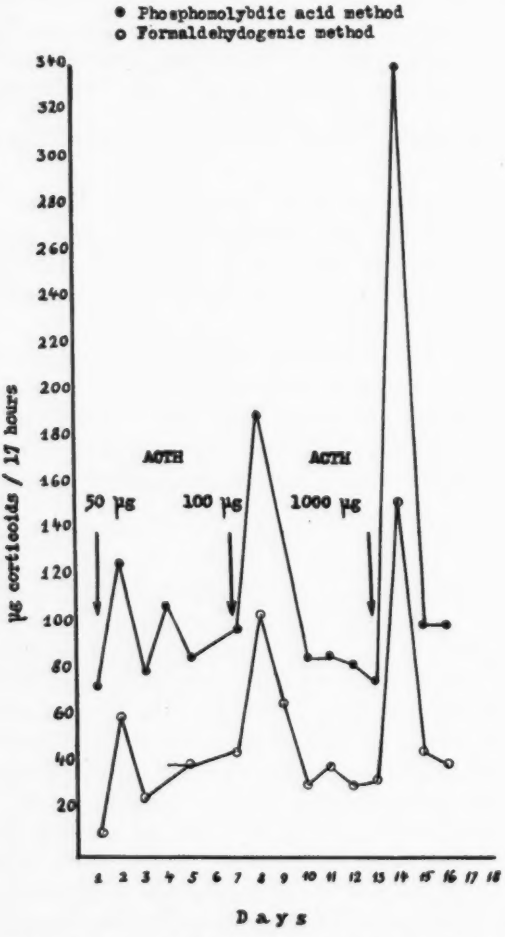

FIG. 7. Corticoid excretion pattern in the spayed guinea pig administered ACTH.

shown by one of us (Zondek 1931, 1941, 1944, 1946) small doses of estrogenic hormones exert a stimulating effect on the anterior pituitary, whereas large ones cause inhibition. It is possible by suitable doses of estrogens to stimulate the lactotropic, gonadotropic and thyrotropic hormones and by these means to cause in the rabbit, milk secretion, luteinization of the ovary

and the appearance of thyrotoxic symptoms similar to those seen in Graves' disease in man. In the light of these experiments and of our present results we are of the opinion that estrogens mobilize ACTH in the guinea pig and thus cause increased corticoid excretion. It is apparent that the corticoid excretion does not increase parallel to the estrogen doses. In contrast to this the corticoid increases approximately in parallel with ACTH dosage. It is probable that the estrogenic hormones release the ACTH which is present at the time of administration in the pituitary.

This hypothesis that the estrogens stimulate the adrenal cortex through their action on the pituitary can only be proved by experiments on hypophysectomized guinea pigs.

The relations between the adrenal cortex and ovarian function are known from many studies which have shown that hypertrophy of the cortex occurs during estrus or ovulation or following administration of estrogens, and that atrophy occurs after ovariectomy or following administration of androgens. (For bibliography see Burrows (1949), and Hartman and Brownell (1949).)

CONCLUSIONS

Adrenal cortical function appears to be related to ovarian activity. Urinary corticoids in the guinea pig show a cyclical variation which is closely related to the estrous cycle. During ovulation urinary corticoids increase. Ovariectomy results in a low and acyclical output of corticoids. The pattern of corticoid excretion in the male guinea pig is the same as that of the ovariectomized female guinea pig. Administration of estrogens causes an almost immediate increase of urinary corticoids in both the intact and spayed female animal. It is assumed that estrogens stimulate the anterior pituitary gland to discharge ACTH.

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PITUITARY-ADRENAL CORTICAL ANTAGONISM TO ESTROGENIC STIMULATION OF THE UTERUS OF THE OVARIECTOMIZED RAT

OBSERVATIONS ON STRUCTURAL SPECIFICITY OF CRYSTALLINE STEROIDS[1]

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IT WAS demonstrated in an earlier report (Szego and Roberts, 1948) that adrenal cortical hyperactivity, induced by adrenocorticotrophin, had a powerful inhibitory influence on the responsiveness of the castrate uterus to administered estrogen. Attempts to reproduce a similar degree of depression with adrenal cortical extract or with certain pure crystalline steroids which were available at that time met with but partial success. The striking inhibition exerted by adrenocorticotrophin was not duplicated by any of the following: Compound A, testosterone, Upjohn's adrenal cortical extract in oil, progesterone, or desoxycorticosterone acetate. The latter two steroids appeared to interfere to some extent with the uterine response to estrogen. However, the degree of inhibition was far short of the virtual obliteration of the response observed with endogenous adrenal cortical hyperactivity induced by adrenocorticotrophin.

In an extension of these observations, it has been possible to demonstrate, as described in the present report, that this complete activity resides in at least two pure steroids: 17-hydroxy-corticosterone (Compound F) and 11-dehydro, 17-hydroxy-corticosterone (cortisone), compounds which have recently been demonstrated to reproduce other manifestations of adrenal cortical hyperactivity (cf., Sayers, 1950). At least one of these (Compound F), has been demonstrated to be secreted *in vivo* (Nelson, Reich and Samuels, 1950; Reich, Nelson and Zaffaroni, 1950; Nelson *et al.*, 1951).

The present report deals with an evaluation of some crystalline steroids related to adrenal and/or gonadal activity with respect to the property of interference with the uterine response to estrogen.

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MATERIALS AND METHODS

Most of the procedures employed have been described in detail in earlier reports (Szego and Roberts, 1945, 1948; Roberts and Szego, 1947). Young adult virgin rats of the Sprague-Dawley strain were used approximately two weeks after ovariectomy. Some of the earlier experiments were performed on animals of the Wistar strain.

Hypophysectomized Sprague-Dawley animals and their controls were obtained from a commercial dealer[2] and shipped via Air Express 3 days after operation. They arrived in good condition and were subjected to ovariectomy approximately 2 days after arrival. None of these animals died as a result of ovariectomy, but some losses occurred during the subsequent 2-week period which preceded the experiments; these losses could be ascribed to pneumonia and occurred in spite of careful regulation of environmental temperature. Those animals which came to experiment were in good condition after maintenance on a powdered ration consisting of $\frac{2}{3}$ ground Rockland pellets and $\frac{1}{3}$ modified Steenbock diet (Lindow, Peterson and Steenbock, 1929), fortified with 6% Mazola oil and supplemented by lettuce and cod-liver oil. Completeness of hypophysectomy was ascertained by gross inspection of the sella turcica at autopsy.

During the experimental period the rats were permitted free access to water and Rockland laboratory chow. In the case of hypophysectomized animals, the powdered ration was continued. Intravenous solutions were administered under light ether anaesthesia via the saphenous vein in the amount of 0.5 ml. per 100 grams body weight. These included control saline, estradiol-17 β[3] in physiological saline (cf., Roberts and Szego, 1947, for details of preparation), and saline suspensions of the following steroids, prepared as described below: 17-hydroxy-corticosterone[4] (hereafter called Compound F), Compound F-acetate,[5] 11-dehydro, 17-hydroxy-corticosterone acetate[6] (hereafter called Compound E-acetate), Δ5-6 pregnenol-3 β,-one-17 (hereafter called pregnenolone), 11-desoxycorticosterone acetate,[7] testosterone,[8] and progesterone.[9] Additional animals received, intravenously, adrenal cortical extract (aqueous, Wilson's) in the amount of 0.5 or 1.0 ml. per 100 grams body weight, or ACTH (Actarmour) 0.25 ml. ($\cong$2.5 mgm. L A-IA standard) in saline per 100 grams body weight. Estrogen was administered at zero time; all other materials were injected immediately following. The uteri were removed exactly four hours after the administration of estrogen, and weighed in both the wet and dry state as described in previous communications from this laboratory. The maximal uterine water inhibition response (Astwood, 1938) has been found to occur at this time in the control ovariectomized rat (Szego and Roberts, 1948).

Where terminal blood was required for related analytical procedures, the animals were exsanguinated from the dorsal aorta under light ether anaesthesia.

Preparation of the crystalline steroid suspensions in saline was facilitated by a sug-

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[2] Hormone Assay Laboratories, Chicago, Illinois.

[3] Estradiol-17β was generously provided by the Schering Corporation.

[4,5] A sample of pure crystalline Compound F was made available through the courtesy of Dr. Peter Forsham, who also donated a vial of Upjohn's Compound F-acetate suspension in saline. An additional sample of pure crystalline Compound F-acetate was received through a generous contribution from Dr. Harold Mason.

[6] Compound E-acetate suspension in saline (Cortone) was made available through the generous cooperation of Dr. Hans Molitor of the Merck Company.

[7,8,9] Dr. Ernst Oppenheimer of the Ciba Corporation graciously placed at the disposal of the investigator generous amounts of pure crystalline 11-desoxycorticosterone acetate, testosterone and progesterone.

gestion from Dr. Harold Mason (1951) that a small amount of synthetic detergent be added. Accordingly, the requisite weighed amount of steroid was ground in an agate mortar with a few drops of physiological saline and one drop of a commercial detergent.[10] The contents of the mortar were transferred quantitatively to a 10 ml. glass-stoppered graduated cylinder with repeated rinsings of saline and made up to volume. The final concentrations were in most instances 2.5 mgm. steroid per ml. These suspensions were prepared fresh for each experiment, and were well agitated immediately before injection.

Where commercial preparations of steroids in aqueous suspension were available (Compound E-acetate and Compound F-acetate) they were diluted to the required concentration with physiological saline without recourse to additional detergent. Excellent suspensions were thus available in all cases, and were well tolerated with the exceptions noted below. It may be noted that by comparison the steroids administered in the earlier study (Szego and Roberts, 1948) were in relatively more unstable suspension.

RESULTS

A. *Ovariectomized Rats*

Crystalline steroids of adrenal origin (Compounds F and E) and structurally related compounds (desoxycorticosterone acetate and pregnenolone) were administered intravenously in saline suspension as described, immediately following the intravenous injection of estradiol. In addition, as studied in the earlier series (Szego and Roberts, 1948) testosterone and progesterone were again included in the present observations for two reasons: first, because they are known to antagonize certain estrogenic effects (Robson, 1939; Astwood, 1939, 1940) and secondly, because of the improved physical state of suspension available as compared to the earlier studies in this laboratory.

In Table 1 are summarized the results with these materials.

It will be noted first, that the control animals without estrogen treatment (Group 1) exhibit the relatively uniform castrate uterine water levels observed earlier (Szego and Roberts, 1945; Roberts and Szego, 1947). The table further reveals that an increase of a high degree of statistical significance is brought about in four hours by intravenous administration of estrogen (Group 2). The uterine water response was demonstrated to be maximal with the dose of 0.5 μgm. per 100 grams body weight (Szego and Roberts, 1948).

Compound F-acetate at a dosage of 1.25 mgm. per 100 grams body weight may be observed to interfere in a striking manner with the degree of uterine water imbibition noted in the animals receiving estrogen alone ($p < 0.001$). On the other hand, this dose of Compound F-acetate did not,

[10] One drop of undiluted "Tween-80" (Atlas; polyoxyethylene sorbitan monooleate) was used in a final total volume of 10 ml. The proportion of detergent was reduced appropriately when smaller volumes of steroid suspension were prepared.

as noted in the statistical portion of the table, reduce the uterine response to the castrate level entirely ($p=0.001$). Such inhibition of estrogen response was, however, achieved by doubling the dose of Compound F-acetate administered:[11] under these circumstances, the treated animals

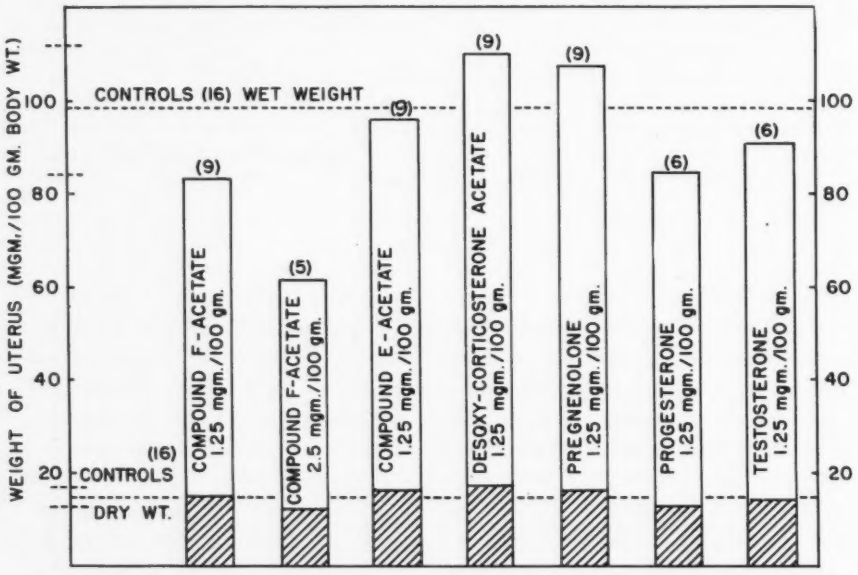

FIG. 1. *The Influence of Crystalline Steroids on the Uterine Response to Intravenously Administered Estradiol.* The total height of the bars represents the average uterine wet weight for each group; the cross-hatched portion, uterine dry weight. The dotted lines locate the corresponding average values obtained in control animals; their respective standard deviations are indicated by marked intercepts on the left-hand ordinate. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses. All animals were injected with 0.5 micrograms of estradiol-17 β per 100 grams body weight in addition to the treatment described for each group in the figure. Autopsy was performed four hours later. The untreated control values are as follows: wet weight uterus, 57.5 ± 9.2; dry weight, 11.8 ± 1.8, mgm. per 100 grams body weight, respectively.

were indistinguishable from the castrate controls in uterine water content ($p=0.2$).

[11] It may be noted that while the higher dose level of 2.5 mgms. per 100 grams body weight was well tolerated by the animals receiving Compound F-acetate, the equivalent dose of cortisone acetate killed several rats in respiratory collapse before it was discontinued. At autopsy, lungs of the latter group were hemorrhagic, and may have contained emboli of crystals. An estimate of crystalline size in the original suspensions was not obtained; however, gross differences in stability were not discernible.

TABLE 1. INFLUENCE OF CRYSTALLINE STEROIDS ON THE UTERINE WATER, RESPONSE OF THE OVARIECTOMIZED RAT TO ESTROGEN[1]

Group	Treatment[2]	Dose (mgm./100 gm. body wt.)	No. of rats	Per cent uterine water[3]	"p" value[4]	
					vs Group 1	*vs* Group 2
1	Control	—	7	79.5 ± 0.6 (78.6 to 80.5)		
2	Estrogen alone	—	16	84.5 ± 0.8 (83.2 to 85.6)	<0.001	
3	Estrogen + Compound F-acetate	1.25	9	81.5 ± 1.1 (79.0 to 83.3)	0.001	<0.001
4	Estrogen + Compound F-acetate	2.50	5	80.3 ± 1.1 (78.6 to 82.1)	0.2	<0.001
5	Estrogen + Compound E-acetate	1.25	9	82.6 ± 0.9 (80.6 to 84.3)		<0.001
6	Estrogen + Desoxycorticosterone acetate	1.25	9	84.4 ± 0.6 (83.6 to 85.4)		0.9
7	Estrogen + Pregnenolone	1.25	9	84.5 ± 0.5 (83.5 to 85.3)		—
8	Estrogen + Progesterone	1.25	6	84.3 ± 0.5 (83.8 to 85.3)		0.6
9	Estrogen + Testosterone	1.25	6	84.0 ± 2.0 (80.3 to 86.8)		0.2
10	Estrogen + ACE, Wilson's aqueous	0.5–1.0 ml.	10	83.1 ± 1.5 (79.1 to 84.8)		<0.01

[1] Estradiol-17β(0.5 micrograms per 100 grams body wt.) was injected intravenously into all animals except Group 1 four hours before autopsy.
[2] The crystalline steroids were administered intravenously in saline suspension (see text) immediately after estrogen injection.
[3] Mean, standard deviation and range are shown.
[4] Calculated by the Fisher "t-test."

As seen with the 1.25 mgm. dose of Compound F-acetate, Compound E-acetate at that dose level reproduced the antagonism to the uterine response to estrogen which had been observed with ACTH earlier ($p < 0.001$). It would appear from the range of the uterine water content, however, that cortisone acetate was somewhat less effective on either an absolute or a molar basis in this respect than was Compound F-acetate. Similar observations on relative effectiveness are recorded in Figure 1 when the criterion of total uterine weight per 100 grams body weight is used. These will be discussed below.

Table 1 further reveals that desoxycorticosterone acetate treatment

(Group 6) in no way affected the uterine response to estrogen ($p = 0.9$). Similarly, the animals treated with pregnenolone, progesterone and testosterone exhibited no diminution in their capacity to react to simultaneously administered estradiol (cf., Groups 7, 8, 9 *vs.* Group 2). The uterine water values in the estrogen+testosterone group, however, were more variable than seen in other experiments and an occasional depressed response was seen (cf., range). Adrenal cortical extract (Wilson's aqueous) administered intravenously in dosage from 0.5 to 1 ml. per 100 grams body weight exerted a moderate damping effect on the uterine response ($p < 0.01$). The effectiveness of adrenal cortical extract was erratic, however. In some animals, complete obliteration of the uterine response was effected (uterine water 79.1%); in others, no apparent influence on the estrogen-induced water imbibition could be detected. It will be recalled, however, that earlier attempts to reproduce the antagonism phenomenon with pretreatment of the animals with adrenal cortical extract in oil (Upjohn's) had been negative (Szego and Roberts, 1948). Thus, a partial effect with aqueous material intravenously administered indicates that the problem may be solved by adequate study of route and dosage. This is especially likely in view of the fact that the maximal dose of 1 ml. utilized was equivalent to only 0.3 to 0.4 mgm. of Compound E.

Figure 1 depicts the responses of these groups as measured by the criteria of wet and dry uterine weights relative to body weight. It has been observed many times in these and similar experiments (Szego and Roberts, unpublished data) that the criterion of uterine water percentage is the more reliable in terms of reproducibility. Thus, it will be observed from the figure that while both doses of Compound F-acetate produced a definite depression of the total uterine weight per 100 grams of body weight as compared with the response of animals receiving estrogen alone, the variability of the controls on this basis precludes the degree of statistical significance observed when actual water content is used as a criterion. Consequently, only the 2.5 mgm. dose of Compound F exhibits an unequivocal degree of depression of the uterine response on a weight basis. Moreover, it is apparent that the profound degree of interference with water imbibition seen with Compound E (Table 1) is not reflected in a significant decrease in total uterine weight (Figure 1). It appears also from the figure that depression of uterine weight without significant alteration in water content (Table 1) could be elicited with progesterone. Thus, dissociation of effects in modifying the estrogen response with respect to total weight and composition can be discerned.

B. *Hypophysectomized, Ovariectomized Rats*

It appeared of interest in the course of these investigations to consider

the responsiveness of the hypophysectomized preparation, both with respect to estrogen sensitivity alone, and also in terms of the capacity of certain representative steroids to alter the uterine response.

Table 2 summarizes results with the hypophysectomized preparation. Two ovariectomized but otherwise intact groups with and without estrogen treatment are also included for comparison. It will be observed first, that the uterine water content of the untreated hypophysectomized, ovariectomized rat (Group 3) and its appropriate non-hypophysectomized

TABLE 2. THE INFLUENCE OF HYPOPHYSECTOMY ON THE UTERINE RESPONSE OF THE OVARIECTOMIZED RAT TO ESTROGEN[1] WITH AND WITHOUT ADRENAL STEROIDS

Group	Treatment[2]	No. of rats	Uterine weight (mgm./100 gm. body wt.[3])		Uterine water		
			Wet	Dry	Per cent[3]	Statistical evaluation	
						vs. group no.	"p" value[4]
	Non-hypophysectomized						
1	Control	7	57.5 ± 9.2 (44.9 to 71.1)	11.8 ± 1.8 (9.0 to 13.9)	79.5 ± 0.6 (78.6 to 80.5)		
2	Estrogen alone	16	98.2 ± 13.8 (74.1 to 120.0)	15.1 ± 2.2 (10.1 to 18.6)	84.5 ± 0.8 (83.2 to 85.6)		
	Hypophysectomized						
3	Control	5	45.1 ± 5.6 (38.2 to 55.3)	9.6 ± 0.8 (8.8 to 11.1)	78.6 ± 1.0 (77.0 to 79.8)	1	>0.1; <0.2
4	Estrogen alone	4	77.3 ± 8.1 (64.5 to 86.4)	12.1 ± 1.9 (9.4 to 13.4)	84.4 ± 0.9 (82.9 to 85.4)	2	>0.8; <0.9
5	Estrogen + Compound F-acetate	4	49.7 ± 5.6 (44.4 to 58.9)	10.1 ± 0.9 (9.1 to 11.1)	79.8 ± 1.1 (78.2 to 81.2)	3 4	0.2 0.001
6	Estrogen + Desoxycorticosterone acetate	3	75.8 ± 10.9 (61.7 to 88.2)	12.7 ± 1.5 (10.7 to 14.3)	83.2 ± 0.7 (82.8 to 83.7)	4	>0.1; <0.2

[1] Estradiol-17β (0.5 micrograms per 100 grams body weight) was injected intravenously into all animals except Groups 1 and 3, four hours before autopsy.
[2] Where additional steroid administration is indicated, the materials were injected intravenously in saline suspension (see text) immediately after the estrogen.
[3] Mean, standard deviation, and range are shown.
[4] Calculated by the Fisher "t-test."

control (Group 1) are indistinguishable ($p>0.1$). This indicates that hypophysectomy is not attended by alterations in degree of uterine hydration. Moreover, assessment of the influence of the hypophysis in altering the capacity of the animal to respond to intravenous estradiol (Groups 4 *vs.* 2) reveals that no quantitative differences exist ($p>0.8$). Thus similar baselines may be used for comparison throughout the series. It may be noted in passing that vaginal sensitivity to administered estradiol had been shown to be unaltered up to 20 days following hypophysectomy of the ovariectomized rat (Del Castillo and Di Paola, 1942).

Table 2 further demonstrates that Compound F-acetate is perhaps even more effective in the hypophysectomized animal than in its control, for it is apparent that the 1.25 mgm. dose per 100 grams body weight exerts an influence powerful enough to obliterate the estrogen response completely (Group 5 *vs.* the estrogen-treated hypophysectomized Group 4), $p=0.001$;

this set of animals is indistinguishable from the untreated hypophysectomized control (Group 3): $p = 0.2$. It will be recalled that twice the dose of Compound F-acetate was necessary to produce a similar degree of inhibition in the animals with pituitaries intact (Table 1). As in the previous experiments with ovariectomized but otherwise intact rats, desoxycorticosterone acetate did not produce a statistically significant depression in the response of hypophysectomized animals to intravenous estradiol ($p > 0.1$).

DISCUSSION

The data presented reveal that Compound F and cortisone possess to a striking degree the capacity to reproduce the inhibition of the uterine water response to estrogen which had been noted earlier with augmented adrenal cortical activity following adrenocorticotrophin administration. This phenomenon has been demonstrated in the absence of the pituitary. Improvements in technical aspects of administration of the steroids by the intravenous route permitted the demonstration of estrogen-antagonistic effects within four hours. Absorption from intramuscular injection, it may be added, was too slow within this period to permit demonstration of Compound E antagonism; on the other hand, Compound F exerted a moderate inhibitory effect when given by this route. These, and other data presented above, indicate that Compound F was consistently the more effective substitute for endogenous adrenal cortical hyperactivity. The striking failure of desoxycorticosterone, pregnenolone, progesterone, testosterone, and Compound A (Szego and Roberts, 1948) to influence the uterine response to estrogen in a consistent and reproducible manner was further evidence for the structural specificity of the 21-hydroxy compounds in this regard. It is of interest to note that pregnenolone, which has been considered as one possible source material for the biosynthesis of Compound F as evidenced by conversion *in vitro* in the presence of surviving adrenal tissue (Hechter *et al.*, 1951) was ineffective in the present study. The demonstration of the importance of at least one of the 21-hydroxy, 11-oxygenated steroids which were effective in the present study from the point of view of adrenal secretory activity *in vivo* (Nelson, Reich and Samuels, 1950; Reich, Nelson and Zaffaroni, 1950; Nelson *et al.*, 1951) and *in vitro* (cf., Hechter *et al.*, 1951) together with their capacity to reproduce many of the effects of ACTH in these and other studies (cf., Sayers, 1950; Ingle, 1950; Conn *et al.*, 1951), lends further support to the physiological significance of the present findings. Failure to reproduce as complete an estrogen inhibition with adrenal cortical extract as was obtained with cortisone and Compound F does not appear to be a serious handicap to the interpretation of the present study. It is difficult to assess the factors of dosage and relative concentration of the several hormonal activities present in the complex extract in terms of absolute equivalence.

Failure in the present experiments to observe a clear-cut depressing influence of progesterone, desoxycorticosterone and testosterone upon the estrogen-induced uterine water response seems difficult to interpret in view of the demonstrated effects of these compounds in interfering with the vaginal cornification activity of estrogen (Robson, 1939, 1950; Del Castillo and Di Paola, 1942; Dohan *et al.*, 1951). The slight inhibitory effect of desoxycorticosterone and of progesterone on uterine water imbibition seen in an earlier report from this laboratory (Szego and Roberts, 1948) was not confirmed in the present report when observations were extended to include larger numbers of animals. Notwithstanding these primarily negative data, an occasional animal did reveal depression in total uterine weight when progesterone was given simultaneously with estrogen (cf., Astwood, 1940), without, however, notable depression of uterine water content. The greater variability of the data on progesterone and testosterone suggest that conditions of dosage and time and the criteria used in assessing interference with an estrogen effect may be of importance in the interpretation of these results. The occasional dissociation of effect on uterine weight increase and water content alluded to earlier, may underlie these difficulties in interpretation.

The obvious implication of the present findings, in view of the dependence of the uterine response upon shifts in water and electrolytes, is that the effective steroids antagonized the underlying osmotic equilibria (Talbot *et al.*, 1940) associated with the estrogen response through their well-known capacity to influence salt and water metabolism, perhaps even lowering initial water content of the uterus so that the estrogen would have to exert its effect on a lower baseline. This possibility seems invalid for several reasons: first, the conclusive failure of desoxycorticosterone, and second, the lack of significant influence of Compound F alone, without estrogen, upon the castrate uterine water content: (78.6 ± 0.5 *vs.* 79.5 ± 0.6 in the controls). It may be noted that an independent control, determination of liver water, used in several experiments, demonstrated a consistent lack of variability, and independence of steroid treatment, with or without estrogen, in confirmation of previous studies by Carroll (1945). Extensive observations on the simultaneous changes in water content of other tissues, notably muscle, however, have been made by Zuckerman and co-workers (1939, 1950), and reveal widespread shifts among tissues following estrogen, progesterone and desoxycorticosterone administration to the rat.

It was thought possible in the present experiments that osmotic changes secondary to alterations of organic metabolism by the individual steroids might be reflected in a rise in osmotic activity of the serum, thus effectively preventing the net uni-directional water movement required for the uterine response. This possibility was explored briefly through the measure-

ment of the conductivity of some representative samples. Preliminary observations indicated, however, no differences among animals receiving estrogen with or without Compound F-acetate or dexosycorticosterone acetate with respect to untreated controls. In addition, determination of serum total and non-protein nitrogen did not reveal differences among similar groups at the 4-hour interval used in the present study. In view of these negative data, therefore, it is difficult to ascribe the inhibitory influence of cortisone and Compound F purely to a specific interference with the osmotic salt-water balance whether direct, or secondary to other changes.

The present data lend themselves to interpretation from another viewpoint, however, namely that the 11-oxy, 17-hydroxy steroids which were specifically effective in the inhibition of the estrogen response had in common the capacity to affect some fundamental process in organic metabolism of the uterine cell. While the first concrete evidence of estrogen action on the uterus has been demonstrated to be an increase in osmotically active particles preceding, in point of time, the water imbibition (Talbot, Lowry and Astwood, 1940; Cole, 1950), as well as the rise in Q_{O_2} (Kerly, 1940; Carroll, 1942), it is apparent that even the osmotic alterations may be secondary to changes in tissue enzyme activity. While the present investigation was in progress, related studies in this laboratory (Beyler and Szego, 1951) the details of which will be reported in a separate communication, revealed that Compound F-acetate and cortisone acetate exerted a powerful inhibitory influence upon the capacity of intravenously administered estrogen to augment β-glucuronidase activity of the uterus and certain accessory reproductive structures in the castrate rat. Four hours following the administration of these compounds simultaneously with estrogen as described in the present study, the β-glucuronidase activity remained at the castrate level, whereas that of the estrogen-treated controls was significantly increased. Desoxycorticosterone acetate was incapable of altering the estrogen response. The consistent correlation with the uterine water findings in the present report is strongly suggestive of the possibility that structural specificity of the steroid may be involved in influencing some enzyme system (not necessarily β-glucuronidase), whose activity is fundamental to the alterations in organic metabolism which accompany early uterine growth (or upon which subsequent cell division may depend for energy sources). A growing literature on the effects of cortical steroids on enzyme systems is becoming available (cf. Ingle, 1950 and Lieberman and Dobriner, 1951). The relation of cortical steroids to the activity of some fundamental enzyme systems involving -SH groups has been reviewed (cf., Anderson *et al.*, 1951). Alternatively, appropriate enzyme systems may be influenced by changes in electrolyte environment alone

(cf., Riley, 1950), and thus secondarily by salt and water changes elicited by certain steroids.

Opsahl (1949a, b, c) has made a detailed study of steroidal influence on hyaluronidase activity, and has dealt at length with the problem of permeability. A direct approach to the mechanism of 11-oxy, 17-hydroxycorticosteroid interference with estrogen-induced water imbibition of the uterus by way of permeability alterations in the uterine capillaries seems indicated by these observations, together with the earlier studies by Hechter, Krohn and Harris (1941).

Still another mechanism of interference with estrogen activity which depends upon structural specificity comes to mind, namely, that of competitive inhibition. Specific reference to this subject has been studiously avoided in the literature, except in an occasional instance (Woolley, 1946; Hertz and Tullner, 1950; Arhelger and Huseby, 1951; Deane and Gardner, 1951) for lack of direct evidence. However, it appears justifiable to include mention of this possibility here because the presently described metabolic, and not merely morphological (cf. Del Castillo and Di Paola, 1942; Robson, 1939, 1950; Dohan, 1951) evidence of steroid antagonism if extended, may throw some light on the mechanism of steroid action. The type of competitive inhibition referred to is that of competition by Compound F and cortisone and, potentially, compounds of similar configuration, for a specific protein which may otherwise be essential for estrogen transport and activity (Szego and Roberts, 1946a, b; Roberts and Szego, 1946, 1947), and which, by serving as a steroid carrier, may be effectively of enzyme or coenzyme nature. An analogous situation for desoxycorticosterone and the apoenzyme of D-amino acid oxidase has been described (Hayano *et al.*, 1950). This approach is being subjected to critical examination in our laboratories at the present time. Preliminary evidence at hand suggests that Compound S (17-hydroxy, 11-desoxycorticosterone) acetate,[12] and Compound D (allopregnane-3β,-17"β", 21-triol, -11, 20 dione)[12] also possess a moderate capacity to inhibit estrogen-induced uterine water imbibition. This suggests that the 17-hydroxy group, together with an oxygen function at position 3 of the steroid nucleus separated by the appropriate intramolecular distances, may be the requisite structural factors involved in the antagonism to the polar estrogens (cf., Scheuler, 1946), and that the 11-oxy function may be relatively dispensable on a qualitative basis.

Studies on the capacity of these and related steroids to interfere with the stilbestrol-induced uterine water response are in progress (Whitney and Szego, unpublished observations). It has been suggested (Arhelger

[12] Compound S-acetate and Compound D were generously supplied by Dr. A. White, Chemical Specialties, Inc.

and Huseby, 1951; Robson and Sharaf, 1951) that synthetic estrogen in its effects on mammary and vaginal growth, is not as readily antagonized by certain hormonal steroids as is naturally-occurring estrogen.

While the elucidation of the mechanism of adrenal inhibition of estrogen activity would be of fundamental theoretical importance, the phenomenon has immediate applications which are becoming apparent in view of the widespread clinical ACTH and cortisone program (Szego, 1950).

SUMMARY

The previously demonstrated antagonism between pituitary-adrenal cortical hyperactivity and the sensitivity of the rat uterus to estrogenic stimulation has been confirmed. The phenomenon has been reproduced unequivocally by Compound F and cortisone and shown to be independent of the presence of the hypophysis. A moderate influence was observed with adrenal cortical extract (Wilson's, aqueous). Preliminary data suggest that Compounds S and D (Reichstein) possess this capacity to a limited degree. Desoxycorticosterone, pregnenolone, progesterone and testosterone failed to exert the inhibitory effect in a consistent manner.

Possible mechanisms are discussed in the light of the apparent requirement for structural specificity.

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"BIOASSAY OF INSULIN IN SERUM USING THE RAT DIAPHRAGM"

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GEMMILL (1939, 1941) demonstrated that, in the presence of glucose, small quantities of insulin accelerated both the synthesis of glycogen and the utilization of glucose by the isolated rat diaphragm. Many observers have since confirmed and extended his results. Although much interesting and enlightening information has been obtained, few investigators have attempted to utilize this effect for the bioassay of insulin. It was our intention to explore the practicability of applying the technique of Gemmill to the detection of either small quantities of insulin or anti-insulin factors in the sera of humans. Serum containing excess insulin would be expected to increase the utilization of glucose and synthesis of glycogen, while serum containing anti-insulin factors would be expected to decrease the glycogenetic effect of insulin added to the media.

METHODS

In all experiments, the procedure was very similar to that originally described by Gemmill (1939, 1941) and by Stadie and Zapp (1947). Young adult white male rats of the Wistar strain weighing between 100 and 150 grams were used. After a 24 hour fast, they were decapitated. Each hemidiaphragm was then removed and blotted on filter paper. Any obvious blood was carefully pressed out of vascular channels and non-muscular tissue was trimmed away. The diaphragms were then divided into as many pieces as necessary for the individual experiment (usually four). The pieces were each weighed on a torsion balance and placed immediately into Warburg flasks containing the nutrient or test medium with or without added insulin. The flasks were kept in iced trays until all were ready for oxygenation.

First filter paper strips and then 0.2 ml. of 2N NaOH were added to the center wells of the Warburg flasks. The flasks were gassed with 100% oxygen for five minutes and then shaken for two hours in the Warburg bath kept at a temperature of 37.5 degrees C. They were then removed from the bath, and the muscle strips were placed in 1 ml. of 30% KOH for glycogen analysis. One ml. of the nutrient medium was taken for glucose analysis. When the initial glycogen content of the diaphragm was to be determined, one weighed portion of the muscle was placed in a test tube containing 1 ml. of 30%

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KOH immediately after removal from the animal. Glucose utilization and/or glycogen synthesis were determined and are reported as mg. glucose/100 mg. of wet tissue. In each case, the insulin effect was the difference between the glucose used or glycogen synthesized by the insulin-exposed portion(s) of diaphragm(s) as compared with the non-insulin-exposed portion(s) of diaphragm(s) of the same animal or group of animals. Glucose in the medium was determined by the method of Nelson (1944), glycogen in the muscle by the method of Good, Kramer and Somogyi (1933).

Nutrient medium, consisting of either 2 ml. Krebs' Ringer-phosphate (KRP pH 7.4) containing 0.3% glucose or a mixture of 1 ml. of human serum containing approximately 0.1% glucose and 1 ml. of KRP solution containing 0.5% glucose, was used in each Warburg. Thus, in the latter instance, the final glucose concentration was approximately 0.3% at the beginning of the experiment. When glucose utilization was studied, the glucose concentration of the mixture was carefully determined both before and after the two hour incubation period.

The insulin used was regular insulin (Squibb). The amount used varied with the experiment. When an experiment involved giving insulin to a patient and also adding it to the Warburg flasks, the same lot of insulin was used. The insulin was diluted with distilled water so that only 0.1 ml. of the insulin solution had to be added to the two ml. of medium in the Warburg flask to give the final concentration of insulin desired.

Two variants of the basic technique were used. Early in the work, the generally accepted procedure was followed, i.e. the dipahragm of each animal was divided into four approximately equal pieces, and each portion put separately into a Warburg flask containing the medium to be tested. Each experiment required only one animal, one section of diaphragm serving as a control for other sections from the same diaphragm. For each specimen of serum to be tested, at least three rats were utilized simultaneously, the experiment thus being run in triplicate. We shall henceforth refer to this as the "single diaphragm" method.

The other method, called the "pooled diaphragm" technique, was suggested by Willebrands, Groen, Kamminga and Blickman (1950). We adopted it later in the work to attempt to eliminate the animal variation which we encountered. Quarter-diaphragms from six rats were pooled together in four Warburg flasks so that finally each of the four Warburg vessels contained six pieces of diaphragm, one from each rat. One experiment thus utilized six animals.

The amount of glucose used by a single diaphragm during an experiment was calculated as the difference between the amount of glucose originally placed in and that finally remaining in the Warburg flask. The magnitude of the difference was small as compared with the values for the latter two, a statistically undesirable situation. Greater accuracy was therefore obtained in the single diaphragm method by measuring glycogen content and synthesis in the diaphragm as an indication of insulin effect. However, in the pooled diaphragm method, the six strips of muscle utilized a considerable part of the available glucose. Therefore it was practical to use glucose utilization as the measure of insulin activity.

Analysis of the data in the literature collected by Perlmutter and Greep (1948) concerning the effect of insulin upon glucose utilization and glycogenesis indicated that glycogenesis accounted for an average of 80% (range 57–104%) of the increase in glucose utilization. Therefore the data on glucose utilization and glycogenesis as measured by the diaphragm methods are not completely comparable. One would expect a greater quantitative effect upon glucose utilization than upon glycogenesis.

RESULTS

For many months after the commencement of this work, while attempting to do control studies, our results were extremely inconsistent from day to day and from animal to animal. We often failed to reproduce the classical results, i.e. increased glycogen synthesis or glucose utilization upon the addition of adequate amounts of insulin. In an attempt to rule out possible obstructing factors, we repeated the experiments with the following modifications: 1. adrenalectomy of the rats; 2. injection of epinephrine $\frac{1}{2}$, 1 or 3

TABLE 1. EFFECT OF VARYING QUANTITIES OF INSULIN ON GLYCOGEN SYNTHESIS

Insulin concentration	Number of experiments	Mean insulin effect	Standard error of mean	't' value
units per ml.		mg. glucose per 100 mg. of wet tissue		
0.005	13	0.117	0.014	8.6
0.01	16	0.128	0.023	5.6
0.1	14	0.206	0.017	12.4

Single diaphragm technique using K.R.P. solution with 0.3% glucose. Insulin effect expressed as increment of glycogen synthesized over the control.

hours before decapitation in order to decrease the initial glycogen content of the diaphragm; 3. use of other strains of white rats; 4. use of the same strain which another group of investigators* was using at that time; 5. use of the diaphragms of newborn rabbits. None of these changes in technique brought about more consistent results. Because the insulin effects were most variable during the summer months, we compared the results from the diaphragm of rats kept in an air conditioned room with those of litter mates kept at summer room temperature. There was no difference in the lack of insulin effect. Finally, without known change in conditions or technique, the results became more consistent and the insulin effects reported in Table I were then obtained. We mention all this in order to emphasize how variable this technique can be.

Effect of Varying Doses of Insulin on Glycogenesis

Using the "single diaphragm" method and KRP solution with 0.3% glucose as the medium, the effect of varying quantities of insulin on glycogen synthesis was determined (Table 1). Even with concentration as low as 0.005 unit of insulin per ml. of medium, the mean insulin effect was statistically significant. In a series of experiments the insulin effect increased with increasing amounts of added insulin. However, it was not possible to estimate the amount of insulin present from the result of a single experi-

*From Dr. Stadie's laboratory in the University of Pennsylvania.

ment since one half of a given diaphragm might show a small insulin effect in the presence of a larger concentration of insulin than the other half which was exposed to less insulin.

Effect of Human Serum on Glycogenesis of Rat Diaphragm

The glycogen synthesis in a mixture of 50% human serum and 50% KRP solution and in 100% KRP solution were compared both with and without added insulin and the results in the two media were the same. Thus it was concluded that normal human serum does not contain enough insulin or anti-insulin factors to be detected by this technique. When the sera from different human subjects were compared, no detectable differences were noted.

Single Diaphragm Method

The next series of experiments was planned to answer these questions: 1. What was the variability of glycogen synthesis by the "single diaphragm" method in media containing human serum without added insulin? 2. Having determined the standard deviation of the method what could be considered a significant insulin effect in a single experiment in our laboratory? 3. Would the "single diaphragm" method be able to detect the difference between the insulin content of human serum before and after the intravenous injection of insulin?

The procedure of these experiments was as follows: After withdrawing a sample of fasting blood, 20 units of insulin[1] were injected intravenously into human subjects. Venous blood was drawn from the opposite arm five minutes later.[2] The blood was allowed to clot and after centrifugation, the serum was removed. The sera from the two specimens of blood were each mixed with equal volume of KRP solution containing 0.5% glucose. In all experiments, the diaphragms were placed in the media containing the human serum, within 90–120 minutes after the blood was withdrawn from the donor patient. In these experiments, the diaphragms of the rats were

[1] 20 units of insulin were chosen as the dose to be injected because of the following calculations: 20 units mixed in a serum pool of 2500–3000 ml. would equal a concentration of .007–.008 units of insulin/ml. of serum. Since this serum is diluted with an equal portion of KRP solution, the final concentration is approximately .004 units/ml. of medium. Table 1 illustrates that our technique was able to detect concentrations of insulin of this order of magnitude. Stadie and Zapp (7) reported similar findings.

[2] It was assumed that five minutes was enough time for the insulin to be distributed throughout all the plasma, but not enough for the insulin to be attached to any tissue or to affect carbohydrate metabolism in the recipient. We also assumed that any demonstrable insulin effect produced by the serum obtained after the intravenous insulin injection could be attributed to the insulin which was injected.

each divided into four pieces. One piece was used for a control and was placed in flask 1 which contained 2 ml. of the diluted fasting human serum. The second piece was placed in flask 2 which contained 2 ml. of the diluted serum, which had been drawn five minutes after the intravenous injection of insulin. The third piece was placed in flask 3 which, in addition to 2 ml. of diluted fasting serum, contained insulin added directly to the Warburg flask. The fourth piece was used either as a duplicate of flask 1 (in order to study the variation of glycogen synthesis without added insulin) or similar to flask 3 except that it was exposed to a concentration of insulin

TABLE 2. EFFECT OF INCREMENT IN SERUM INSULIN, AFTER INTRAVENOUS INJECTION OF INSULIN, ON GLYCOGEN SYNTHESIS—"SINGLE DIAPHRAGM" METHOD

Insulin concentration	Number of experiments	Mean insulin effect	Standard error of mean	't' value	Standard deviation
units per ml.		mg. glucose per 100 mg. of wet tissue			
0	50	0.001	0.007	0.1	0.046
*	43	0.112	0.015	7.4	0.099
0.005	33	0.116	0.017	6.8	0.092
0.1	23	0.166	0.016	10.4	0.081

Single diaphragm technique using equal parts of K.R.P. solution with 0.5% glucose and of normal human serum. Insulin effect expressed as increment of glycogen synthesized over control.

* Concentration resulting from mixture of equal parts of K.R.P. solution with 0.5% glucose and of serum obtained 5 minutes after the intravenous injection of 20 units of insulin into normal human subjects.

different from that for the third piece (in order to study the effect of varying doses of added insulin).

The subjects used in these experiments were free of any disease known to affect carbohydrate metabolism. There were no untoward reactions to the insulin, as the subjects were given breakfast as soon as the second sample of blood was drawn. The concentration of glucose in the serum, drawn five minutes after the intravenous injections of insulin, did not differ from the fasting pre-injection glucose level. The results of these experiments are summarized in Table 2.

Variability of Single Diaphragm Method

When a duplicate determination of flask I was run, the mean difference in a series of 50 experiments was only +0.001 mg. of glycogen per 100 mg. of rat diaphragm. The standard deviation of this series was ±0.046 mg. of glycogen per 100 mg. of diaphragm. Considering two standard deviations as the range which would include 95% of the results, it was decided to designate as significant any synthesis of glycogen varying from the control by more than 0.09 mg. per 100 mg. of rat diaphragm.

Efficacy of Single Diaphragm Method in Detecting Increase in Insulin Content of Serum

In a series of 43 experiments, in which the glycogen synthesis in flask 1 was compared with the synthesis in flask 2, the mean effect of the intravenously injected insulin was +0.112 mg. of glycogen per 100 mg. of diaphragm. Since the standard error of the mean was ±0.015, the t value was 7.4. This is a statistically significant increase in glycogenesis which we assume was due to the injected insulin. The mean insulin effect in this series (+0.112) is almost identical with the insulin effect (+0.116) caused by

TABLE 3. EFFECT OF INCREMENT IN SERUM INSULIN, AFTER INTRAVENOUS INJECTION OF INSULIN, UPON DIAPHRAGM SENSITIVE TO DIRECTLY ADDED INSULIN AND UPON DIAPHRAGM RESISTANT TO DIRECTLY ADDED INSULIN—"SINGLE DIAPHRAGM" METHOD

Reaction of diaphragm to added insulin	Number of experiments	Mean insulin effects	Standard error of mean	't' value	Number & % sensitive to intravenous insulin
		mg. glucose per 100 mg. of wet tissue			
Sensitive	29	0.142	0.015	9.5	23 (79%)
Resistant	14	0.049	0.027	1.8	4 (29%)
Total	43	0.112	0.015	7.4	27 (63%)

Single diaphragm technique using equal parts of K.R.P. solution with 0.5% glucose and of normal human serum. Insulin effect expressed as increment of glycogen synthesized over control.

the addition of a theoretically comparable amount of insulin (i.e. 0.005 units per ml. of medium) directly into the Warburg flask.

Analysis of the individual experiments, studying the insulin effects from the sera obtained after the intravenous injection of insulin, revealed a wide scatter of results. Many experiments failed to reveal an insulin effect. A possible explanation for the wide scatter was individual variation in the sensitivity of the rat diaphragm to insulin. Therefore, the 43 experiments were classified into two groups as determined by the effect of either 0.005 or 0.1 unit per ml. of insulin added to the Warburg (flask 3). If the glycogen synthesis in flask 3 exceeded that in flask 1 by at least 0.09 mg. per 100 mg. of diaghragm, the diaphragm was considered insulin sensitive; if the synthesis was less than 0.09 mg., the diaphragm was considered insulin resistant. When the 43 experiments were thus subdivided, 29 rat diaphragms were insulin sensitive and 14 were insulin resistant (Table 3).

It will be noted in Table 3 that 23 (79%) of the 29 rat diaphragms, which were sensitive to added insulin, were also sensitive to the slight increase in serum insulin content resulting from the intravenous injection of 20 units of insulin.

The 14 rat diaphragms which were insensitive to added insulin were also, as a whole, insensitive to the small increment of serum insulin produced by

the intravenous injection of 20 units of insulin. Only four of the 14 diaphragms (29%) responded to the sera by significant insulin effects.

The mean insulin effect of the 14 experiments, +0.049 mg., was significantly different from the mean insulin effect of the 29 sensitive diaphragms, +0.142 mg. This added validity to the idea that in one group, the animals were insensitive indicators of the presence of insulin and results from them should be discarded rather than considered negative. Thus, to a considerable extent, it was possible to decrease the scatter of the results.

Lack of Effect of Human Serum on Insulin Action

Previously, we stated that early control studies failed to demonstrate an anti-insulin or insulin-like effect in human serum. Table 4 demonstrates

TABLE 4. EFFECT OF NORMAL HUMAN SERUM UPON GLYCOGEN SYNTHESIS INDUCED BY VARYING CONCENTRATIONS OF INSULIN

Insulin concentration	Number of experiments	Medium	Mean insulin effect	Standard error or mean	't' value
units per ml.			mg. glucose per 100 mg. of wet tissue		
0.005	13	K.R.P.	0.117	0.014	8.6
0.005	33	K.R.P. +serum	0.116	0.017	6.8
0.1	14	K.R.P.	0.206	0.017	12.4
0.1	23	K.R.P. +serum	0.166	0.016	10.4

Single diaphragm technique using medium with final glucose concentration of 0.3%. Insulin effect expressed as increment of glycogen synthesized over control.

that the addition of human serum had no effect upon the increased glycogenesis caused by the direct addition of 0.005 unit of insulin per ml. of medium. The apparent greater effect of 0.1 unit of insulin per ml. of KRP medium compared to its effect in serum-KRP medium is not statistically significant. The results from the serum-KRP medium were obtained in the experiments just cited.

Pooled Diaphragm Method

In order to further eliminate animal variation and the necessity to discard results from insensitive animals, the "pooled diaphragm" variant of the technique was tested. Twenty units of insulin were injected intravenously as described in the previous experiment. Glucose utilization by the six pooled quarters of the diaphragms was measured setting up the four flasks as in the single diaphragm method. Table 5 presents the data thus obtained.

Variability of Pooled Diaphragm Method

The mean glucose utilization of control flask 1 differed from the mean

utilization of a duplicate flask by 0.02 mg. per 100 mg. of diaphragm in 16 experiments. This mean was not significantly different from zero. Since the standard deviation was 0.05 mg., 95% of the control studies would be expected statistically to be less than 0.10 mg. This was therefore considered to be the critical level; any insulin effect greater than this was considered significant.

Efficacy of Pooled Diaphragm Method in Detecting Increase in Insulin Content of Serum of Normal Subjects

Of 25 experiments in which the "pooled diaphragms" were tested for sensitivity to insulin added directly, only one failed to respond with an in-

TABLE 5. EFFECT OF INCREMENT IN SERUM INSULIN, AFTER INTRAVENOUS INJECTION OF INSULIN, ON GLUCOSE UTILIZATION—"POOLED DIAPHRAGM" METHOD

Insulin concentration	Number of experiments	Mean insulin effect	Standard error of mean	't' value	Standard deviation
units per ml.		mg. of glucose per 100 mg. of wet tissue			
0	16	0.02	0.012	1.7	0.05
*	24	0.17	0.017	10.0	0.08
0.005	16	0.21	0.020	10.5	0.08
0.1	16	0.30	0.030	10.0	0.13

Pooled diaphragm technique using equal parts of K.R.P. solution with 0.5% glucose and of normal human serum. Insulin effect expressed as increment of glucose utilized over control.
* Concentration resulting from mixture of equal parts of K.R.P. solution with 0.5% glucose and of serum obtained 5 minutes after the intravenous injection of 20 units of insulin into normal human subjects.

TABLE 6. COMPARATIVE FREQUENCY OF INSULIN RESISTANCE AS MEASURED BY "SINGLE DIAPHRAGM" AND "POOLED DIAPHRAGM" TECHNIQUES

Insulin concentration	Diaphragm method	Number of experiments	Number & % resistant to insulin
units per ml.			
0.005	Single	33	17 (52%)
0.005	Pooled	16	1 (6%)
0.1	Single	23	3 (13%)
0.1	Pooled	16	0 (0%)

sulin effect which exceeded 0.10 mg. per 100 mg. of diaphragm. Thus 4% of the "pooled diaphragm" experiments had to be discarded for insensitivity as compared with 33% of the "single diaphragm" experiments.

The data presented in Table 6 indicates that the "pooled diaphragms" are less apt to be insulin resistant at either concentration of insulin. It also appears that the resistance of the "single diaphragm" to insulin is a relative phenomenon, since resistance is encountered four times as frequently at the lower concentration of insulin.

Of the 24 experiments in which the "pooled diaphragms" were sensitive

to added insulin, 18 (75%) demonstrated a significant increment of glucose utilization in the flask containing serum drawn after intravenous insulin. This figure is very similar to the 70% of the sensitive "single diaphragms" which were able to demonstrate the increase in serum insulin content following intravenous insulin administration.

Efficacy of Pooled Diaphragm Method in Detecting Increase in Insulin Content of Serum of Diabetic Patients

The identical experiment, using the "pooled diaghragm" method, was repeated using the sera from a group of mild diabetic patients, who were

TABLE 7. EFFECT OF SERUM FROM MILD DIABETIC SUBJECTS AS COMPARED TO EFFECT OF NORMAL HUMAN SERUM AS TESTED BY "POOLED DIAPHRAGM" TECHNIQUE

Type of serum	Insulin concentration	Number of experiments	Mean insulin effect
	units per ml.		mg. glucose per 100 mg. of wet tissue
Normal	0.005	16	0.21
Diabetic	0.005	15	0.25
Normal	0.1	16	0.30
Diabetic	0.1	15	0.33
Normal	*	24	0.17
Diabetic	*	15	0.24

Pooled diaphragm technique using equal parts of K.R.P. solution with adequate glucose to make final concentration 0.3%, and of either diabetic or normal serum. Insulin effect expressed as increment of glucose utilized over control.

* Concentration resulting from mixture of equal parts of K.R.P. solution and of serum obtained 5 minutes after the intravenous injection of 20 units of insulin into either normal or diabetic subjects.

not receiving insulin prior to the experiment. The average fasting blood sugar was 185 mg.%. The results in Table 7 reveal that the sera of mild diabetics behave like the sera of normal subjects both in the presence of intravenously injected insulin or in the presence of insulin added directly to the medium. There was no evidence of insulin-inhibiting substances present in their sera. No experiment had to be discarded for insensitivity of the diaphragms to insulin added directly. Thirteen of the 15 experiments (87%) showed detectable insulin in the serum after the intravenous injection of 20 units of insulin.

Effect of Intravenously Administered Glucose Upon "Insulin Content" of Human Serum

The next experiment was planned to determine whether the rapid intravenous injection of 25 grams of glucose could cause an increment in the serum insulin content which might be detected by the "single diaphragm" technique. Thirteen such experiments were performed in which the glucose

was injected within a four minute period. Sera was drawn before the glucose injection and 15, 30, 45, or 60 minutes after the injection. Since the glucose content of the serums varied, adjustments were made in the glucose content of the KRP diluting solution, so that the final glucose concentration was 0.3% in every flask. Table 8 reveals that the mean increase of glycogen synthesis over the control flasks were less than 0.09 mg. per 100 mg. of diaphragm, thus no increase in insulin content was noted at any of the times tested.

TABLE 8. EFFECT OF INTRAVENOUSLY INJECTED GLUCOSE UPON INSULIN CONTENT OF NORMAL HUMAN SERUM AS DETECTED BY "SINGLE DIAPHRAGM" TECHNIQUE

Time after glucose injection	Insulin added	Number of experiments	Mean increment of glycogen synthesis over control	Mean blood sugar
minutes	units per ml.		mg. glucose per 100 mg. of wet tissue	
0	0.1	25	0.168	87
15	0	8	0.032	200
30	0	15	0.024	151
45	0	16	0.027	135
60	0	10	0.026	121

Effect of Administered ACTH or Cortisone

In order to determine whether the administration of ACTH or cortisone produced an anti-insulin substance in the serum, the sera of 8 patients who were receiving this type of hormonal therapy were tested by either the single or pooled diaphragm technique. Five of the eight sera did not have any anti-insulin effect; in the other three experiments, the added insulin had no glycogenetic effect. These data are too few for critical analysis.

Three experiments were performed in which 0.1 unit of insulin per kilogram of body weight was injected intravenously into human subjects. The amount of insulin injected was 7 units in two subjects and 3.5 units in the third. The sera of all three subjects drawn five minutes after intravenous administration of the insulin contained an increment of insulin which was demonstrated by the diaphragm technique.

A single experiment was performed in which 0.05 units of insulin per kilogram of body weight was injected intravenously, the amount of insulin being 2.5 units. The serum of this patient, drawn five minutes later, did not demonstrate any detectable increase in insulin content.

A most important clinical application of the diaphragm technique would be its ability to detect abnormally large concentrations of insulin in the sera of patients having functioning islet cell tumors of the pancreas. We did not have the opportunity to adequately test the serum of any patient who was proved to have this malady. During the early part of our investigation, the serum from a patient with a proved islet cell tumor was tested by

the "single diaphragm" technique. This patient's serum did not differ from the sera of normal subjects in stimulating glycogen synthesis. This failure may have been due to the resistance of the rat diaphragm, since at that time the addition of 0.1 unit of insulin per ml. of medium failed to stimulate glycogenesis.

DISCUSSION

The experimentalist has been able to induce diabetes mellitus either by surgical removal of the pancreas or chemical injury of the islet tissue, thus decreasing the availability of insulin to the body, or by the injection of anti-insulin hormones such as ACTH or growth hormone. Similar results have been observed in human subjects. Diabetes caused by pancreatectomy is relatively easily controlled by small doses of insulin, these patients being insulin sensitive; diabetes brought about by ACTH or growth hormone is difficult to control even with large doses of insulin, these patients being insulin resistant. However, it has not been possible to determine whether diabetes mellitus, which is not associated with other endocrinopathies, is due to a deficiency of insulin or to an excess of anti-insulin factors. The ability to measure the serum content of insulin and of anti-insulin factors would therefore be helpful in this respect. In addition, it would be of use in differentiating functioning islet cell tumors of the pancreas from other causes of hypoglycemia. This may be difficult to do with diagnostic measures presently available.

Many investigators have attempted to measure the insulin content of serum. Gellhorn *et al.* (1941) injected blood into hypophysectomized adrenodemedullated rats, and measured the fall in blood sugar. They reported that the insulin content of normal human blood drawn in the fasted state, was approximately 0.2 milliunits per ml. They noted that in two patients who had symptoms of hyperinsulinism, the serum insulin concentration exceeded the normal concentration. Bornstein (1950) injected serum into alloxan diabetic, hypophysectomized, adrenalectomized rats and measured the drop in blood sugar. He concluded that the concentration of insulin in normal human serum was 0.1 milliunit per ml. He also noted that, after the oral ingestion of glucose, the serum contained two to three times more insulin than the fasting serum.

Mirsky *et al.* (1948) determined the daily excretion of insulin in the urines of normal and diabetic subjects and in a subject having an islet tumor. The amounts excreted were minute, but the mild diabetic excreted an amount of insulin approximately half that of the normal subjects. A patient with an islet cell tumor of the pancreas, reported by Willner and Weinstein (1950), excreted approximately nine times as much insulin as

normal subjects. Mirsky's method of preparing the urine for analysis was quite intricate; the assay was done by measuring the decrease in blood sugar of rabbits as a result of the injection of the urine extract.

Although these methods seem sensitive enough, they are much too complicated for use in the laboratories of even well equipped hospitals. Therefore, it was hoped that the clinical application of the diaphragm method might be useful in the development of a simple assay for serum insulin and anti-insulin factors. Apparently others have worked along similar lines. Spoont and Dyer (1951) reported that the serum of a patient, in an insulin resistant phase of severe diabetes mellitus, markedly reduced the effect of insulin as demonstrated by the diaphragm technique. They also found normal or diabetic serum only slightly decreased the effect of added insulin. These latter results are similar to our own.

Our data indicate that the diaghragm technique, in our laboratory, was unable to detect the concentration of insulin present in normal human serum. It was also unable to demonstrate any increment in serum insulin during the hour after intravenous injection of 25 grams of glucose. However, our data do demonstrate that the diaphragm method can detect approximately 5 milliunits of insulin per ml. of serum. Neal *et al.* (1950) using the same rat diaphragm technique were able to detect approximately 4–5 milliunits of insulin per ml. of media, but not 1 milliunit. If Gellhorn's (1941) and Bornstein's (1950) independent estimates of fasting serum insulin content be correct (0.1–0.2 milliunits/ml.), and if after oral glucose, this value is increased only two or threefold, then the rat diaphragm technique, at least as used in our laboratory, is too insensitive to detect insulin in serum of the fasting human subject or after glucose administration. Thus, our findings do not disagree with those of Bornstein. On the other hand, Willebrands, Groen, Kamminga and Blickman (1950) using the pooled diaphragm technique, reported that they could detect as little as .005 milliunits of insulin per ml. of medium.

The diaphragm technique can detect an increment in the insulin content of serum drawn after the intravenous injection of 20 units of insulin. Our calculations indicate that if 20 units were dispersed in approximately three liters of serum, there would result a concentration of approximately 5 milliunits of insulin per ml. of serum. At this concentration of insulin, no anti-insulin factors could be detected either in the serum of normal human subjects or of mild diabetic patients who were not receiving insulin therapy. Although we desired to test the serum of severely diabetic subjects, it was not considered advisable to deprive these patients of insulin therapy for the two or three days necessary to be certain that all exogenous insulin had disappeared.

The lack of patients with hyperinsulinism prevented us from determining whether the diaphragm method would be sensitive enough to detect the increase in serum insulin content which probably is associated with functioning islet cell tumors. Studies of the serum of patients with islet cell tumors by means of the "pooled diaphragm" technique should be performed.

SUMMARY AND CONCLUSIONS

An attempt was made to adapt the Gemmill technique to clinical use by eliminating the factor of animal insensitivity. The "pooled diaphragm" technique is superior to the "single diaphragm" method in this respect.

Normal and diabetic sera do not alter the results of this technique as obtained in Krebs' Ringer-phosphate medium.

The elevation in serum insulin content produced by the intravenous injection of 20 units of insulin, can be detected 5 minutes after the intravenous injection.

25 Gm. of glucose injected intravenously do not cause the secretion of sufficient insulin to be detected by this technique.

Limitations of the method and possible directions for future investigation are discussed.

ADDENDUM:

Since this paper was submitted for publication, Marsh and Haugaard (*J. Clin. Invest.* **31**: 107–110, Jan. 1952) have reported that, by using the Stadie dipping modification of the Gemmill technique, the sera of normal subjects and of mild diabetics contain demonstrable anti-insulin factors. Willebrand *et al.*, in the same journal (*J. Clin. Invest.* **31**: 97–106, Jan. 1952), reported that, by using the multiple diaphragm technique, they demonstrated the presence of detectable amounts of insulin in the sera of normal subjects. If both these reports are corroborated, it would appear that the Gemmill technique can be useful to the clinician. However, it should be pointed out that there is an apparent contradiction in these two reports. Using substantially the same technique, the former investigators report an anti-insulin effect while the latter report an insulin effect in normal human serum.

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THE HORMONE CONTROL OF OVULATION IN THE CALF

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CASIDA *et al.* (1943) demonstrated that follicular development could be stimulated in the ovaries of the immature calf by treatment with anterior pituitary extracts. It was therefore thought possible that further investigation might offer a practical method of obtaining a supply of cow ova for further experimentation.

METHODS

Heifer calves of varying ages were injected subcutaneously twice daily with an anterior pituitary extract high in F.S.H. (follicular stimulating hormone). This treatment was continued from 72 to 96 hours after which an interval of 48 hours was allowed when the animals were intravenously injected with human chorionic gonadotrophin or pituitary extracts high in L.H. (Luteinising Hormone). The animals were killed 72 hours after the intravenous injection.

RESULTS

The results almost immediately indicated a great predominance of follicular development over luteinisation (Fig. 1). However, one or more ovulations were observed in 55% of the animals treated though the number of ovulated or luteinised follicles was always greatly inferior to the number of large unovulated follicles. J. Hammond, Jr. and Bhattacharya (1943) showed that the mature cow could be superovulated by a subcutaneous injection of P.M.S. (Pregnant Mare Serum) and that ovulation would generally occur spontaneously under the influence of the animal's own pituitary. Early in the experiment it was noted that even at three weeks after birth the gonadotrophin stimulated calf could ovulate spontaneously without the aid of an intravenous injection of a luteinising preparation. These ovulations were few in number, generally being one or two, while the number of apparently fully developed follicles often exceeded 40. The results from these experiments (Table 1) compare favourably with those of Casida *et al.* who, while using animals of unknown age (veal calves) obtained 129 follicles but only 9 corpora lutea (9 animals treated). It was thus presumed that a high percentage of ovulations did not occur in the immature calf, due to the infantile state of the animal's own pituitary. It was there-

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FIG. 1. Ovaries removed from a calf six weeks after birth following a single six day treatment with pituitary gonadotropins.

fore decided to give similar treatment to a series of calves of increasing ages in an endeavour to determine the stage of maturity at which superovulation could be obtained.

Two calves, 9 and 17, are of particular interest because, though each received identical treatment with the animals listed in Table 1, their ovaries were markedly different. Instead of being predominantly unovulated follicles they were largely composed of luteal tissue.

Calf No.	Age	Unovulated follicles	Fresh corpora lutea
9	8 weeks	17	49
17	14 weeks	27	45
		44	94

Considerable difficulty was experienced in explaining this marked difference which occurred in both ovaries in each case from two animals

TABLE 1

Calf No.	Age	Undeveloped follicles	Fresh corpora lutea
29	7 days	4	0
28	9 days	13	3
30	2 weeks	0	0
1	3 weeks	65	1
2	3 weeks	47	2
3	4 weeks	1	0
4	4 weeks	26	0
39	6 weeks	58	2
35	7 weeks	9	2
5	8 weeks	27	4
7	9 weeks	38	0
8	9 weeks	32	1
12	12 weeks	3	0
15	12 weeks	21	3
13	13 weeks	3	0
26	14 weeks	11	1
41	15 weeks	79	0
25	19 weeks	20	0
31	32 weeks	38	15
19	44 weeks	17	1
20 calves		512	35

The above calves received a single treatment of Anterior Pituitary Injections as described in the text.

treated at different times of the year and of quite different ages. It was considered possible that in these two cases a follicle of considerable size may have been present at the beginning of the experiment and that this follicle luteinised rapidly during the pituitary treatment. Hammond (1927) has reported follicles approaching mature size in calves from 4 to 12 weeks after birth and the writer has occasionally observed follicles over .5 cm. in diameter in the newly born calf. Rowson (1951) has also reported an increase in the percentage of follicles ovulating in cattle treated with P.M.S. during the luteal phase of the cycle. Further, Cole and Miller (1933), Thibault and Laplaud (1948) and Robinson (1950) have all demonstrated that in anoestrous sheep superovulation could best be achieved either by P.M.S. or anterior pituitary extract if the initial treatment was repeated in from 13 to 16 days. These observations suggest the importance of the role played by luteal activity and it was thus considered that experiments involving pre-treatment and the establishment of an "experimental corpus luteum" might help in obtaining a greater number of ovulations from the immature calf as well as providing useful information on the mechanism of ovulation.

A new series of experiments was then begun in which the treatment previously used was repeated twice with an interval of 15 days between treatments. This interval was so arranged that by the time any follicles stimu-

lated had reached maturity, the "experimental corpus luteum" was 21 days old, thus simulating the normal cycle of the mature cow.

In each case when the ovary reponds to a second gonadotrophic treatment in the presence of a waning corpus luteum from the first series of in-

TABLE 2

Calf No.	Age	Unovulated follicles	Fresh corpora lutea
47	6 weeks	5	33
48	8 weeks	0	33
56	12 weeks	1	53
37	28 weeks	10	8
		16	126

The above calves received a 6-day pre-treatment with the establishment of an experimental corpus luteum and a second identical pituitary treatment 15 days later.

jections there occurs multiple ovulation of the majority of follicles stimulated (Fig. 2). The term "stimulated follicles" is applied to those follicles which obtain a diameter of not less than .7 cm. and can be easily counted macroscopically on the surface of the ovary. In each animal treated throughout the experiments the results have always been constant in

FIG. 2. Ovaries removed from a young calf following a second treatment with pituitary gonadotropins as described in the text.

both ovaries. Either the ovaries were a mass of follicles as in the case of animals treated once, except for the two exceptions noted, or a mass of multiple corpora lutea in the cases where the animals were treated a second time.

All these results strongly suggest that the corpus luteum is of prime importance in obtaining complete ovulation when there are many follicles present, as in the case of animals whose ovaries have been stimulated by the injection of gonadotrophins.

RECOVERY OF OVA

One point of interest is the speed with which ova descend in the fallopian tube. Hamilton and Laing (1946) found that in the cow the ovum normally reached the uterus in about 96 hours after ovulation and is in the 8 to 16 cell state. Similar results have been obtained from calves of varying ages following the induction of ovulation by gonadotrophins. The rate of tubular descent, as far as could be concluded by the recovery of ova from the fallopian tubes or uterus, indicates that age has little if any effect on the speed of tubular descent. On the other hand, when an "experimental corpus luteum" has been established and a second anterior pituitary treatment given, the ova are all in the uterus within 48 hours after ovulation. Dowling (1949) observed a similar rapid descent of ova in cattle which had been injected with P.M.S. in the luteal phase of the cycle, 4 to 10 days after oestrus, and in which the corpus luteum was squeezed out 4 days later. Dowling believed, however, that this accelerated descent of the ova down the fallopian tubes was due to the large number of unovulated follicles which he often observed and described as being "over-stimulated."

The results obtained in calves can not support this theory, for even when only one ovulation has been obtained out of from 40 to 60 follicles stimulated, the tubular descent of the ova has remained normal, the ova being recovered from the fallopian tube 72 to 80 hours after ovulation. On the other hand, when a second treatment has been given in the presence of an "experimental corpus luteum" none of the ova obtained has been found in the fallopian tubes even though one of the animals was sacrificed only 24 hours after ovulation. It is concluded that the rapid descent of the ova in the fallopian tube is a direct result of the corpus luteum and not due to the great follicular development which may occur with large doses of P.M.S. or anterior pituitary extract.

SUMMARY

Anterior pituitary injections can produce follicular development in the calf as early as the first week after birth.

The responsiveness of the ovaries of calves to gonadotrophins remains

about the same for a considerable period after birth. The ovaries of calves three weeks old respond as well as those of calves 20 weeks of age.

Ovulation can occur spontaneously in the calf at less than three weeks after birth, provided the follicles have been stimulated by gonadotrophic treatment. However, the number of ovulations is small when compared with the number of large follicles developed.

Intravenous injection of H.C.G. (Human Chorionic Gonadotrophin) or Anterior Pituitary extracts high in L.H. will not substantially increase the number of ovulations obtained from calves receiving only one series of injections.

Superovulation with the formation of multiple corpora lutea can be obtained in the immature calf by repeating the original pituitary treatment after an interval of 15 days has been allowed between treatments.

The rate of descent of ova in the fallopian tube in the calf is not substantially influenced by age, but is greatly accelerated by the presence of an active corpus luteum.

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ON THE NATURE OF THE INTERDEPENDENCE OF THE ADRENAL CORTEX, NON-SPECIFIC STRESS AND NUTRITION IN THE REGULATION OF NITROGEN METABOLISM*

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INTRODUCTION

IT IS now widely recognized that the adrenal cortex is concerned in some fundamental way with the ability of the organism to respond to injury in a characteristic fashion. However, the precise mechanism by which the steroids of the adrenal cortex participate in the metabolic response to injury still remains speculative. Earlier studies in this area led to the concept which is still widely held that certain of the metabolic alterations after injury in the healthy organism are a direct consequence of hypersecretion of adrenal cortical steroids (Selye 1946). However, later work (see Engel, 1951a and b for a review of the literature) has cast doubt on such a simple causal relationship and relatively few investigators active in this field accept this concept any longer. The best evidence against it is found in the report of Ingle, Ward and Kuizenga (1947) who showed that the characteristic negative nitrogen balance which follows fractures in normal, but not in salt-maintained adrenalectomized rats, occurs in the latter if given a constant maintenance dose of adrenal cortical extract. From this came the present working concept that the adrenal cortex is necessary but not responsible for certain metabolic reactions, the negative nitrogen balance of injury being an example.

Previous reports from this laboratory (Engel, Schiller and Pentz 1949; Bondy, Engel, Farrar, 1949), in which the rate of formation of urea nitro-

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*Portions of these data have been presented before the American Association for the Advancement of Science, New York, New York, December, 1949 (Engel 1951a), the 6th Laurentian Hormone Conference, Franconia, New Hampshire, September, 1950 (Engel, 1951b), the 2nd Clinical ACTH Conference, Chicago, Illinois, December, 1950 (Engel 1951c) and the Gordon Research Conference on Food and Nutrition, New London, New Hampshire, August, 1951.

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gen in the nephrectomized rat (Engel, Pentz and Engel, 1948) was used as a sensitive measure of nitrogen metabolism, have presented data concerning the site of action of the adrenal cortex in nitrogen metabolism and factors modifying this action. Evidence has been adduced to indicate that the major action of adrenal hormone in the fasting animal appears to be either to increase the rate of protein breakdown or to inhibit protein synthesis, or both, resulting in a net increase in urea formation. This action of adrenal hormone could be abolished or sharply decreased by the intravenous administration of glucose or amino acids but not by albumin, plasma protein or a fat emulsion. In the adrenalectomized animal intravenously administered whole protein appeared to be less well utilized catabolically than in the normal rat. No clear-cut effect of either adrenal hormone or adrenal insufficiency on amino acid metabolism could be detected in the fasted animal. However, in the presence of a plethora of glucose it has been found recently that urea formation from injected amino acids was decreased in adrenalectomized animals and increased in ACTH treated rats compared to normal animals receiving amino acids and glucose (Engel 1951d).

A hint that the above described changes in nitrogen metabolism in fasted rats treated with adrenal hormone may not necessarily represent direct consequences of hormone action alone is found in the report that insulin hypoglycemia not only tremendously magnified the increase in the urea formation rate which followed treatment of the nephrectomized rat with adrenal extract, but also accelerated the time of occurrence of this increase in nitrogen metabolism (Engel, 1949). Thus, whereas an increase in blood urea characteristically becomes measurable only between 3 and 6 hours after an injection of adrenal extract, if insulin is supplied simultaneously with the adrenal extract, the increase in nitrogen metabolism becomes apparent immediately, e.i., within 3 hours. Insulin alone in the dosage employed had no effect on urea formation during this time. If the hypoglycemia was prevented by glucose administration, the nitrogen response to ACE was abolished. The demonstration that insulin hypoglycemia modifies the metabolic response to adrenal hormone both in time and magnitude lead to the present studies on the influence of various types of stress on the nitrogen metabolism of nephrectomized rats treated with adrenal hormone.

METHODS AND MATERIALS

Except where otherwise indicated, these are the same as in the aforementioned reports from this laboratory in which the rate of urea formation in the nephrectomized rat was used as a measure of nitrogen metabolism during brief consecutive experimental periods. Briefly, the technique involves performing a bilateral nephrectomy about 18 hours before the actual experiment is begun, the animal being fasted during this period.

Blood urea nitrogen levels (Archibald *et al.* 1945) are then measured during appropriate intervals, usually 3 or 4 hours, before and after test substances are injected and the rat of urea formation expressed in mg.N/100./hr., assuming equal distribution of urea throughout the total body water, taken as 63 per cent of the body weight. In over a thousand rats of the strain used in our laboratory, the basal rate of urea formation has been 2.05 ± 0.64[1] mg.N/100gm./hr.

RESULTS

Formalin Stress. In this series of experiments, the acute effect on nitrogen metabolism of a single subcutaneous dose of 4 per cent formalin was studied and compared to that of aqueous adrenal cortical extract (ACE). The first group of animals in Table 1 represent control rats, nephrectomized bilaterally 18 hours previously, which received two consecutive subcutaneous injections of 0.5 ml. of Upjohn's ACE at 0 and 1 hour and urea formation measured from 0–3 hours, 3–6 hours and 6–9 hours. In confirmation of earlier reports from this laboratory a small, but significant increase in urea formation was noted in the second and third 3 hour periods, amounting to 0.68 ± 0.14[2] and 0.57 ± 0.17 mg. urea N/100 grams/hour. When 0.5 ml. of 4% formalin was injected subcutaneously at the end of the third hour in 17 similarly nephrectomized rats (Group 2), no significant change in urea formation occurred in either of the two subsequent periods. However, if ACE was injected by the schedule of Group 1 and then followed by 0.5 ml. formalin, a significant increase in urea formation ensued in both subsequent 3 hour periods (Group 3). The increase was greater than in the ACE treated rats, but only in the last was this difference significant ($p < .02$). These results, which are comparable to the apparent potentiating effect of hypoglycemia on the nitrogen metabolic response to ACE previously reported (Engel, 1949), contrast with the effects of a larger dose of formalin which by itself stimulates nitrogen metabolism. In Group 4, Table 1, it can be seen that 1.0 ml. of formalin subcutaneously at the end of the third hour was followed by an immediate and sustained increase in urea formation amounting to 1.24 ± 0.21 and 0.87 ± 0.20 mg. urea N/100gm./hr. in the 2 subsequent periods. It is important to note, as will be emphasized again later, that the increase in urea formation occurred immediately after the formalin injection in contrast to the delayed response to ACE. When the larger dose of formalin was given to ACE treated animals (Group 5), increases of the order of 1.50 ± 0.31 and 1.16 ± 0.21 mg. urea N/100 gm. per hour occurred. The first was significantly greater than that resulting from ACE alone but neither was different from the effects of 1.0 ml. of formalin. It is clear in this experiment that ACE in this dosage did not potentiate the metabolic response to

[1] Mean ± S.D.

[2] Mean ± standard error, unless otherwise indicated.

1.0 ml. of formalin whereas it did do so when a "subthreshold" dose of formalin was used.

The simplest explanation for the increase in urea N formation after formalin injection is that it is a direct consequence of stimulation of the adrenal cortex by the stress. However, this explanation seems unlikely since the response occurs immediately, in contrast to the delayed effect of ACE and is not modified by simultaneous injections of ACE which, according to Sayers and Sayers (1948), should inhibit ACTH secretion following stress. Further evidence against this mechanism is found in the data from Group 6 and 7, Table 1. In this experiment, two groups of rats

TABLE 1. UREA N FORMATION IN NEPHRECTOMIZED RATS STRESSED BY FORMALIN INJECTION

Group No.	Treatment	No. of rats	Mg. urea N/100 gm./hr.				
			0–3 hrs.	3–6 hrs.	Change	6–9 hrs.	Change
1	1.0 ml. A.C.E.*	27	1.92±0.12	2.60±0.11	+0.68±0.14	2.49±0.13	+0.57±0.17
2	0.5 ml. Formalin†	17	2.08±0.15	2.50±0.19	+0.42±0.23	1.93±0.21	−0.15±0.23
3	1.0 ml. A.C.E.* 0.5 ml. Formalin†	20	1.68±0.11	2.74±0.20	+1.06±0.23	3.02±0.24	+1.34±0.26
4	1.0 ml. Formalin†	21	1.77±0.09	2.91±0.16	+1.24±0.21	2.64±0.22	+0.87±0.20
5	1.0 ml. A.C.E.* 1.0 ml. Formalin†	15	1.74±0.15	3.24±0.22	+1.50±0.31	‡2.87±0.12	+1.16±0.21
6	1 mg. ACTH every 6 hrs. for 24 hrs.	12	3.02±0.19	3.32±0.12	+0.30±0.14	3.22±0.16	+0.20±0.23
7	1 mg. ACTH every 6 hrs. for 24 hr. 1.0 ml. Formalin†	12	2.95±0.24	4.12±0.37	+1.17±0.44	3.97±0.29	+1.02±0.47
8	1.0 ml. Formalin† 0.5 ml. 50% glucose p.o. at 3 hr.	15	2.15±0.17	2.00±0.14	−0.15±0.20	2.30±0.22	+0.15±0.36
9	0.5 ml. 50% glucose p.o. at 3 hr.	7	1.87±0.16	1.57±0.10	−0.30±0.17	2.33±0.17	+0.46±0.32

* 0.5 ml. A.C.E. at 0 and 1 hr.
† Subcutaneously at 3 hrs.
‡ 13 Observations.

were pretreated for 24 hours with 4 mg. ACTH, resulting in a sustained increase in urea formation after nephrectomy. When Group 7 was given 1.0 ml. of formalin at 3 hours, the resulting increases in urea formation during the next two 3 hour periods were identical with those of the rats of Group 4 which received formalin alone and of Group 5 which received ACE and formalin. It is difficult to conceive of the formalin stress stimulating endogenous ACTH secretion in rats already receiving this amount of ACTH. The magnitude of the nitrogen metabolic response to formalin stress thus must be independent of the level of circulating adrenal hormone and not due directly to it, although, as will be confirmed later, a minimum amount of adrenal hormone is necessary for the response. Further evidence against the direct mediation of the pituitary-adrenal axis in this metabolic response is also presented below where it is shown that

the increase in urea formation after massive doses of ACTH intravenously or intraperitoneally occurs only after a delay of 3–6 hours.

Groups 8 and 9 in Table 1 demonstrate that the stimulation of nitrogen metabolism by formalin stress is readily inhibited by glucose, just as was the effect of ACE on nitrogen metabolism (Engel, Schiller and Pentz, 1949).

Confirmation by the nephrectomy technique of the well-known fact that the change in nitrogen metabolism after stress does not occur in the absence of the adrenal cortex is seen in Table 2. Rats were adrenalectomized 5 days before nephrectomy and maintained on a low potassium

TABLE 2. EFFECT OF ADRENALECTOMY ON UREA FORMATION AFTER FORMALIN*

No. of rats	Mg. Urea N/100 gm./hr.		
	0–4 hrs.	4–8 hrs.	Change
15	1.27 ±0.08	1.37 ±0.11	0.10 ±0.12

* 1.0 ml. Formalin injected at 4 hours.

diet, saline drinking water and 0.5 mg. desoxycorticosterone acetate daily (Bondy, Engel and Farrar, 1949). Eighteen hours after bilateral nephrectomy, urea formation was measured for a 4 hour control period and then 1.0 ml. of formalin was injected subcutaneously and urea formation measured for an additional 4 hours. As previously reported, the rate of urea formation was significantly lowered after adrenalectomy and, in contrast to the experiments in Table 1, was not modified by the formalin stress.

Epinephrine Stress. In these studies, a moderately large dose of epinephrine was used as a stress. The dose used, 0.1 mg. per rat subcutaneously, was substantially larger than that necessary to stimulate the pituitary-adrenal axis (Gershberg, Fry, Brobeck and Long, 1950) and presumably its effect was not mediated this way. This type of stress was of particular interest because epinephrine administration was followed by a brisk hyperglycemia. Previously hyperglycemia resulting from glucose injection had been shown to be associated with an inhibitory effect on the nitrogen metabolic response to ACE or to stress. Table 3, Group 1, illustrates the effects of epinephrine on the rate of urea nitrogen formation and on the blood sugar levels (Somogyi, 1945) in nephrectomized rats. A brisk and sustained rise in both are recorded. Noble and Toby (1948) have also reported a negative nitrogen balance in rats after comparable doses of epinephrine. When 0.5 ml. of 50 per cent glucose was given simultaneously with the epinephrine at 3 hours (Group 2), the urea nitrogen change was completely obliterated, blood sugar levels, however, reaching substantially higher levels than in the previous group. It would appear likely that it is

TABLE 3. UREA N FORMATION IN NEPHRECTOMIZED AND ADRENALECTOMIZED NEPHRECTOMIZED RATS STRESSED WITH EPINEPHRINE

Treatment	No. of rats	Mg. urea N/100 gm./hr.					Blood sugar, Mg. per cent			
		0–3 hrs.	3–6 hrs.	Change	6–9 hrs.	Change	0 hrs.	3 hrs.	6 hrs.	9 hrs.
Nephrx	12	1.96 ± 0.14	3.00 ± 0.23	+1.04 ± 0.30	3.20 ± 0.28	+1.24 ± 0.25	—	105 ± 3.1	277 ± 10.5	194 ± 25.8
Nephrx 0.5 ml. 50% glucose p.o. at 3 hrs.	10	2.10 ± 0.25	1.89 ± 0.20	−0.21 ± 0.39	1.97 ± 0.21	−0.13 ± 0.39	—	—	287 ± 26.2	321 ± 50.7
Nephrx-Adrx DOCA-Saline	6	1.27 ± 0.32	0.94 ± 0.17	−0.33 ± 0.33	1.43 ± 0.18	+0.16 ± 0.34	81 ± 7.4	72 ± 6.8	133 ± 4.2	96 ± 11.4
Nephrx-Adrx Cortisone	9	2.87 ± 0.28	3.88 ± 0.24	+1.01 ± 0.27	2.54 ± 0.38	−0.33 ± 0.13	106 ± 2.7	109 ± 5.6	395 ± 53.2	223 ± 53.2

All rats were injected with 0.1 mg. epinephrine subcutaneously at 3 hours.

TABLE 4. EFFECT OF ACTH ON UREA FORMATION IN NEPHRECTOMIZED RATS

Group No.	Treatment	No. of rats	Mg. urea N/100 gm./hr.						
			0–3 hrs.	3–6 hrs.	Change	6–9 hrs.	Change	9–12 hrs.	Change
1	5 mg. ACTH i.v. at 3 hours	5	2.23 ± 0.15	1.79 ± 0.12	−0.71 ± 0.14	2.18 ± 0.18	−0.05 ± 0.28	—	—
2	5 mg. ACTH i.v. for 45 min at 3 hours	4	2.46 ± 0.38	2.11 ± 0.23	−0.35 ± 0.51	3.24 ± 0.16	+0.78 ± 0.39	—	—
3	1 mg. ACTH i.p. at 0, 30, 60, 90 and 120 minutes	9	1.91 ± 0.20	2.64 ± 0.24	+0.73 ± 0.34	3.62 ± 0.17	+1.82* ± 0.25	3.40* ± 0.40	+1.60* ± 0.51

* 7 observations.

the supply of carbohydrate rather than the level of the blood sugar which modifies the nitrogen metabolic response to stress.

When similar studies were performed on desoxycorticosterone and cortisone maintained adrenalectomized-nephrectomized rats, confirmation was made of Ingle's demonstration of the permissive action of adrenal hormone on the nitrogen response to stress. As before, the adrenalectomized rats were kept on a low potassium diet for 4–5 days before nephrectomy. Group 3 were maintained with 0.5 mg. of desoxycorticosterone acetate daily and saline, while the rats of Group 4 were similarly treated until 48 hours before administration of epinephrine and then were given 5 mg. of a saline suspension of cortisone acetate subcutaneously. Twenty-four hours later the cortisone injection was repeated. All rats were nephrectomized in the late afternoon and on the following morning after a 3 hour period 0.1 mg. of epinephrine was injected subcutaneously. Note that in the desoxycorticosterone maintained rats (Group 3) no change in urea formation occurred after the epinephrine, but there was a moderate rise in the blood sugar. The cortisone maintained rats (Group 4) exhibited a significantly higher basal rate of urea formation and fasting blood sugar than did the DOCA maintained rats and after administration of epinephrine showed a rise in urea formation during the subsequent 3 hours which was entirely comparable to that in the rats with intact adrenals (Group 1). However, during the second 3 hour period after epinephrine, the rate of urea formation was no longer increased, a contrast with the control nephrectomized group. Blood sugar rises were quite marked, suggesting that liver glycogen levels were ample. The failure of the increase in nitrogen metabolism to be sustained in the cortisone maintained rats is provocative. It is conceivable that, as suggested by Sayers and Sayers (1948), the rate of hormone utilization is increased during stress and hence that the rate of release of cortisone from the crystalline depot was not adequate to sustain the metabolic response to injury.

SPEED OF METABOLIC RESPONSE TO ACTH

It has already been emphasized that the increase in urea formation in the nephrectomized rat following formalin or epinephrine stress occurred more rapidly than that after small doses of ACE. If the change in nitrogen metabolism after stress were due directly to adrenal steroids resulting from stimulation of ACTH secretion by the stress, one would anticipate that sufficiently large doses of ACTH appropriately administered would have an action at least as rapid as stress. The data of Table 4 demonstrate that this is not the case. In this experiment deliberately massive doses of ACTH were given by the intravenous and intraperitoneal routes to eliminate any criticism that the degree of stimulation of the adrenal cortex by

ACTH might not be comparable in magnitude or degree to that of the stress. In Group 1, Table 4, 5 mg. of ACTH (expressed as Armour Standard LA-1-A) were given in a single intravenous injection after a 3 hour control period. Note that no significant increase in urea formation occurred in the 2 subsequent 3 hour periods. This experiment can be justifiably criticized on the grounds that a single intravenous injection of ACTH, even if massive, may not be comparable to the stimulation of the anterior pituitary by the stress resulting from either formalin or epinephrine. The experiment was therefore repeated with the same dose of ACTH being injected intravenously over a period of 45 minutes. Again no immediate increase in urea formation occurred. If anything there is a suggestive decrease in the rate of urea formation during the first 3 hours in both these experiments. In the second 3 hour interval an increase occurred which was not statistically significant. However, on the basis of experience with this method it is altogether likely that an increase of this magnitude would be significant in a larger series of animals. As a further check and in order to delimit the time and duration of the response to ACTH the hormone was given intraperitoneally in 5 successive injections of 1 mg. each every 30 minutes, beginning at the 0 hour in a third group of rats. Note that the rate of urea formation during the first 3 hours was not different from control periods in previous experiments, that a small but definite increase is noted 6 hours after the first injection and that this increase is clear-cut and sustained during the subsequent 6 hours. These data make it seem most unlikely that the immediate increase in urea formation after stress in the nephrectomized rat can be attributed to endogenous ACTH secretion.

EFFECT OF AMINO ACIDS ON THE NITROGEN METABOLIC RESPONSE TO STRESS

In earlier studies (Engel, Schiller and Pentz, 1949), it was shown that the administration of amino acids seemingly suppressed the accelerated metabolism of nitrogen that followed treatment with adrenal extract. This interpretation was based largely on the fact that the injection of an appropriate amount of amino acid mixture into ACE treated rats was not associated with a greater rate of urea formation than that seen in animals treated with either ACE or amino acids alone. Furthermore, the time pattern of rise in blood urea after combined treatment corresponded to that after injection of amino acids alone rather than that after ACE treatment. In the former case almost all the urea formed in a three hour period could be accounted for in the first hour whereas in the latter approximately equal hourly increments in urea were present during each of the 3 hours. Although no definite evidence could be adduced for an effect of ACE on the

deamination of injected amino acids in the fasted rat in these studies, subsequent observations (Engel, 1951d) indicated that in the presence of a plethora of glucose ACTH treated rats seemingly deaminated a greater proportion of injected amino acids than did normal animals. Table 5 presents the results of observations on the response of stressed nephrectomized rats to amino acid injections. In Groups 2 and 3, 13.1 mg. of amino acid nitrogen per 100 grams body weight were injected intraperitoneally into control and formalin stressed rats. The amino acid mixture used was a 10 per cent solution of the ten essential amino acids plus glycine pre-

TABLE 5. EFFECT OF AMINO ACIDS ON UREA FORMATION IN STRESSED NEPHRECTOMIZED RATS

Group No.	Treatment	No. of rats	Mg. urea N/100 gm. hr.				
			0–3 hrs.	3–6 hrs.	Change	6–9 hrs.	Change
1	1.0 ml. Formalin*	21	1.77±0.09	2.91±0.16	+1.24±0.21	2.64±0.22	+0.87±0.20
2	13.1 mg. Amino Acid N/100 gm. i.p.*	12	1.90±0.13	5.08±0.23	+3.24±0.15	2.42±0.19	+0.52±0.27
3	13.1 mg. Amino Acid N/100 gm. i.p.,* 1.0 ml. Formalin*	9	1.85±0.20	4.74±0.37	+2.89±0.36	3.42±0.36	+1.57±0.46
4	0.4u Insulin i.p. at 0 hr.	14	3.48±0.24	4.09±0.27	+0.61±0.37	5.29±.037	+1.81±0.53
5	0.4u Insulin i.p. at 0 hr. 13.1 mg. Amino acid N/100 gm. i.p.*	13	3.44±0.28	6.33±0.40	+2.89±0.32	6.74±0.42	+3.30±0.47
6	13.3 mg. Amino Acid N/100 gm. i.p.*	12	2.15±0.15	4.37±0.15	+2.22±0.22	2.06±0.11	−0.09±0.22
7	5 days at 4° C. 13.3 mg. Amino Acid N/100 gm. i.p.*	10	3.99±0.23	5.59±0.27	+1.60±0.25	3.86±0.42	−0.13±0.54

* Injected at 3rd hour.

pared and kindly supplied to us by Merck and Co. The control formalin group is the same one reported as Group 4, Table 1. The amino acid injection at the third hour was followed by a sharp increase in urea nitrogen formation equivalent to about 75 per cent of the injected dose. In the second 3 hour period, the rate of urea formation had dropped to the control level. When the same amino acids were given to the formalin treated rats, the resulting 3 hour increase in urea formation was not significantly different from that in the controls receiving amino acids alone, while in the second 3 hour period, the rate of urea formation was the same as expected from formalin alone. These results are quite comparable to those with ACE and amino acids, i.e., there is no additive response in urea formation after amino acid injection and formalin stress. The presumed suppressive effect of amino acid injection did not persist beyond the first 3 hour period.

In Groups 4 and 5, an intraperitoneal injection of 0.4 units of insulin was used as a stress. In contrast with earlier experiments (Engel, 1949) where the insulin was given subcutaneously and a delayed increase in urea formation occurred unless ACE was also administered, the intraperitoneal insulin injection at 0 hour was associated with early hypoglycemia and a change in urea formation during the first 3 hours which progressively increased during the subsequent 6 hours of observation. When the amino acid mixture was given at the third hour, the subsequent increase in urea was about the same during the third to sixth hours as in the unstressed animal given amino acids (Group 2). In the last 3 hours, the rate remained significantly higher than in the insulin treated controls. While the basis for this latter change is not clear, it is again apparent, at least during the period when it is known that injected amino acids are being rapidly metabolized, that the stress of hypoglycemia seemingly did not modify the rate of deamination of the injected amino acids. On the other hand, in contrast with the previous experiment with formalin and earlier experiences with ACE (Engel, Schiller and Pentz, 1949) where amino acid injection seemed to suppress the expected increase in urea formation from formalin or ACE, the amino acid injection in this experiment failed to modify the already established accelerated rate of urea formation resulting from insulin hypoglycemia.

Finally, the chronic stress, 5 days exposure to 4° C. was studied. In this experiment rats were placed in the cold room with food and water ad lib for 100 hours. They were then bilaterally nephrectomized, food removed from the cages, and the animals returned to the cold room for an additional 18 hours. The rate of urea nitrogen formation was measured during a 3 hour control period and found to be 3.99 mg./100gm./hr., (Group 7) a value markedly higher than that in control animals kept at room temperature (Group 6). When 13.3 mg./100grams of an amino acid mixture (a different lot than used in the previous experiments) was injected, the increase in urea nitrogen formation was slightly, but not significantly, less in cold-stressed rats than that in the control animals. Again the chronic stress did not significantly change the rate at which urea nitrogen was formed after an injection of amino acids; and conversely amino acid injection did not suppress the already established elevated basal rate of urea formation resulting from cold stress.

DISCUSSION

From these studies certain facts emerge which are helpful in interpreting previous data on the interrelation between the adrenal cortex and nonspecific stress in nitrogen metabolism. These are as follows:

1. The metabolic response of the nephrectomized rat to non-specific stress differs strikingly from that to ACE or ACTH in that the former is

detectable as an increase in urea formation in 3 hours or less while the latter only becomes apparent in from 3–6 hours.

2. The responses to stress and ACE or ACTH are both suppressed by treatment with glucose or amino acids; although in the case of the most severe stresses, the modification by amino acids is only partial with the doses used.

3. Adrenal hormone is necessary for the metabolic response to stress to occur, as previously shown by Ingle *et al.* (1947); but the magnitude of the response, under the experimental conditions of this study, does not appear to be determined directly by the amount of hormone available, provided a certain minimum quantity is present.

4. In the presence of a plethora of adrenal hormone, the organism responds promptly with an increase in nitrogen metabolism to mildly stressful stimuli which would otherwise have either no or only a delayed effect on nitrogen metabolism.

The results of these studies in general are entirely consistent with the interpretation of the adrenal-stress relationship which has been developed by Ingle and his collaborators (1943, 1944, 1947, 1949, 1951a, 1951b), and has been discussed at some length elsewhere by the present author (Engel 1951b). According to Ingle, the adrenal cortex plays a "permissive" role during stress, being necessary but not responsible for certain metabolic and other changes. This concept is a natural development from the earlier observations of Russell (1939) and of Long, Katzin and Fry (1940) concerning the necessity of the adrenal cortex for the full exhibition of the effects of anterior pituitary extract on carbohydrate metabolism where again the adrenal seems to play a permissive role. It represents a sharp departure from the older views of Selye (1946, 1947), Albright (1943) and Browne *et al.* (1944) which have dominated thinking in this field in the past. According to Selye's original interpretation, certain, but not all, of the metabolic changes which follow non-specific stress were considered a direct consequence of the hypersecretion of adrenal steroids resulting from stimulation of the pituitary-adrenal axis by the stress. Although as early as 1941, Selye and Dosne reported an example of the permissive action of adrenal hormone when they described the hyperglycemic response to traumatic shock of the adrenal extract-treated adrenalectomized animal, the significance of this finding seemingly did not become apparent until recently (Selye, 1950). In earlier discussions of the general adaptation syndrome (Selye 1946, 1947), this observation of Selye and Dosne is not mentioned, and a different interpretation is presented for Ingle's report of the diabetogenic effect of stilbestrol in adrenalectomized animals, maintained on ACE (1943) and the comparable findings of Langley and Clark (1943) that the liver glycogen levels of adrenalectomized animals

exposed to low atmospheric pressure are high when the animals are maintained with ACE, (See Selye, 1946, page 141). In recent publications, Selye (1950, 1951) has altered his interpretation of the adrenal-stress relationship to one which is in essential agreement with that which Ingle and others presented some years ago. Browne (1950) has now emphasized his current belief that a negative nitrogen balance is not necessarily a concomitant of increased adrenal secretory activity in man.

The observation that, in the presence of excess adrenal hormone, the organism responds to minor stresses with changes in nitrogen metabolism comparable to those of more severe stresses, might be considered as an extension of the concept of the permissive role of the adrenal cortex in the response to injury. Adrenal hormone may be looked upon as serving to keep the tissues in a state of preparedness to respond to stress, the level of "alertness" being in part a function of the amount of available hormone. When excess hormone is present, the threshold for the response to stress might be considered as lowered. Overdosage effects of adrenal hormone, then, might be interpreted as hypersensitive and, perhaps inappropriate responses to stimuli which under ordinary circumstances would not be considered sufficiently stressful to elicit significant metabolic changes. Presumably, as emphasized by Ingle (1951a,b) excess hormone released in response to stress serves to maintain homeostasis, whereas the same amount of hormone in the absence of adequate stress results in overdosage effects.

In our experiments, it appears that, provided adequate adrenal hormone is present, the magnitude of the stress determines the protein catabolic response rather than the amount of adrenal hormone for the reaction to stressful stimuli which altered nitrogen metabolism in untreated animals was unmodified by excess ACE or ACTH. A similar observation has been made by Noble and Toby (1948). This finding also supports the concept that the response to stress is not due to hypersecretion of adrenal hormone. Since Sayers and Sayers (1948) have demonstrated that injected ACE or ACTH inhibits endogenous pituitary ACTH secretion, it seems inconceivable that further pituitary or adrenal secretion could have occurred as a result of the stress and hence the increased nitrogen metabolism in these experiments cannot be accounted for through such a mechanism. Further evidence for the primacy of stress in modifying nitrogen metabolism is found in Pearson and Eliel's (1951) report of an increase in the negative nitrogen balance of a patient receiving 17-hydroxycorticosterone when he developed an infection.[3]

[3]Pearson (Personal communication) reports that this patient showed no significant change in urinary steroid excretion during the fibrile episode and exhibited changes in potassium, sodium, chloride, creatine, uric acid and phosphorus excretion in addition to the increase in nitrogen excretion which were identical qualitatively but greater quanti-

Recent studies on carbohydrate metabolism in man (Burns and Engel, 1951) indicate that in contrast to the lack of effect of adrenal hormone excess on nitrogen metabolism during stress noted above, carbohydrate tolerance during stress may be markedly altered in the presence of excess cortisone. Administration of this steroid orally has been found to be associated with a much greater impairment of glucose tolerance in ill than in healthy subjects. Further studies are therefore necessary to establish the precise role of the adrenal cortex in other reactions to injury before generalizations can be made from the study of nitrogen metabolism.

The delayed metabolic response to ACTH described in this report must be placed at the tissue level since the time of response to ACE and ACTH are indistinguishable. Sayers and Sayers (1948) have described metabolic changes in the adrenal cortex within minutes of an injection of ACTH while Nelson *et al.* (1951) have detected significant increases in the level of 17-hydroxycorticosteroids in the blood of humans 15 minutes after an intravenous infusion of ACTH. Both these studies indicate that the adrenal response to ACTH must be exceedingly rapid. The only other investigation we are aware of in which the time of response in nitrogen excretion to ACTH was measured is that of Sayers *et al.* (1949). These investigators noted an increase in urinary nitrogen excretion beginning 3–6 hours after an intravenous infusion of ACTH in normal human subjects. Other evidences of adrenal hormone action, such as eosinopenia, are of course detectable earlier so again one cannot generalize from the nitrogen response.

A comment is in order concerning the essential similarity in the effects of glucose and amino acid treatment on the response to adrenal hormone and stress in these experiments. In the case of glucose, the suppressive effects appear to be identical under the two circumstances while with amino acids there are differences which may be more apparent than real. Thus, while amino acids seemingly suppressed the accelerated nitrogen metabolism from ACE or stress when they were injected prior to the expected increase, they had no effect when urea formation was already increased by stress before amino acid treatment. No comparable experiment has been done with either ACE or ACTH. Likewise no observations have been made on the combined effect of stress and glucose on amino acid deamination comparable to previous studies with ACTH (Engel, 1951d). At present the evidence would suggest that the inhibitory effects of glucose, certainly, and of amino acids, probably, are exerted by a similar mechanism

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tatively than seen after ACTH. Similar events occurred in another patient during fever immediately after withdrawal of ACTH treatment. Again there was no increase in urinary steroid excretion.

during both stress and adrenal hormone activity. These findings then indicate that there are at least three interdependent variables modifying nitrogen metabolism during stress, namely, the stress itself, the adrenal cortex and the nutritive state of the organism at the time of the stress and each of these must be considered in interpreting experiments on stress and/or adrenal hormone action. Although it has not been tested critically under all circumstances, there is a suggestion that the amount of carbohydrate and/or amino acids necessary to modify the response to adrenal hormone and/or stress varies with the dose of hormone and magnitude of stress. In support of this is Werner's (1951) evidence that the negative nitrogen balance following injury may be due primarily to a deficient food intake at this time and Pearson and Eliel's (1951) demonstration of a positive nitrogen balance in a patient with a lymphoma receiving full therapeutic doses of ACTH when his food intake was doubled to conform to his appetite.

It would appear then, that when the nutritive factor is controlled by fasting, small doses of adrenal hormone and modest stresses will readily modify nitrogen metabolism. Since minor stresses likewise accelerate nitrogen metabolism in the presence of excess adrenal hormone, it is conceivable that, since fasting itself is a stress, the effect of adrenal hormone in the fasting animal is apparent only because of the stress of fasting. In the glucose fed animal, the negative nitrogen balance may not occur although ACE still has its usual effects on carbohydrate metabolism (Long, Katzin and Fry, 1940; Engel, Schiller and Pentz, 1949). On the other hand, if the dose of ACTH or adrenal hormone is large enough, negative nitrogen balance will occur with diets on which nitrogen balance ordinarily would be maintained. Is this a response, in the presence of adrenal hormone overdosage, to stresses which ordinarily would not measurably effect nitrogen balance? As indicated above, both this, as well as the response to injury itself, may be abolished if caloric intake is great enough.

SUMMARY

1. Acute stress (formalin subcutaneously, severe insulin hypoglycemia, and epinephrine) causes an immediate increase in urea formation in the nephrectomized rat but not in the adrenalectomized-nephrectomized rat maintained with saline and DOCA.

2. Adrenal cortical extract or ACTH even in massive doses intravenously or intraperitoneally cause only a delayed increase (3–6 hours) in urea formation in the nephrectomized rat.

3. Following mild stress (small doses of formalin or insulin), no immediate change in nitrogen metabolism occurs, but if the animals are treated with adrenal extract an immediate increase in urea formation oc-

curs which is comparable in time of occurrance to that after more severe stresses.

4. Acute or chronic pretreatment with ACE or ACTH does not modify the magnitude of nitrogen metabolic response to a dose of formalin which increases urea formation in the untreated nephrectomized rat.

5. Adrenalectomized-nephrectomized rats maintained on cortisone intramuscularly and acutely stressed with epinephrine exhibit the same prompt increase in urea formation after stress as do animals with intact adrenals. However, the response in the former is not sustained, presumably because of slow release of cortisone from the crystalline depot. It is concluded in agreement with Ingle that adrenal hormone is necessary but not responsible for the nitrogen metabolic response to stress.

6. The concept that adrenal hormone in some way sensitizes the organism to respond to stress with a characteristic metabolic pattern is suggested and discussed. The interdependence of stress, diet and the adrenal cortex in modifying nitrogen metabolism is emphasized.

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THE EFFECT OF CORTISONE ON THE COURSE OF PARABIOTIC HYPERTENSION IN THE RAT*

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SPONTANEOUS evolution of hypertension in rats placed in parabiosis has been described (Hall and Hall, 1951a, b, c). The condition, always confined to one member of a pair, is commonly accompanied by widespread cardiovascular damage consisting of myocarditis, renal arteriolosclerosis and glomerulosclerosis and periarteritis. The frequent occurrence of arthritis in such rats makes the syndrome strikingly similar to that which develops in rats treated with desoxycorticosterone acetate (DCA) (Selye *et al.*, 1943), and suggests that adrenal hyperactivity with respect to mineralocorticoids may be important in the pathogenesis of the syndrome.

There is abundant evidence, both clinical and experimental, relating adrenal cortical hyperactivity and hypertension; some of which has recently been reviewed by Corcoran (1950). It is not surprising therefore that adrenal cortical preparations have been widely used in an attempt to modify the course of both clinical and experimental hypertension, perhaps with a view to depressing adrenal activity.

Perera (1950) has found that cortisone produces a decline in the resting blood pressure of hypertensive patients, although a pressor response may be elicited in the presence of renal disease regardless of whether or not hypertension coexists (Perera, 1951). In this connection it is interesting to note that although cortisone is most effective in preventing the allergic cardiovascular lesions produced in the rabbit by horse-serum (Rich *et al.*, 1950, 1951), it not only fails to prevent similar lesions induced in the rat by anti-rat kidney sera (Hackel *et al.*, 1950), but even aggravates the accompanying hypertension (Knowlton *et al.*, 1949). In the former case cortisone probably interferes in some way with the production of antibodies to horse serum; whereas in the latter case, in which pre-formed antibodies are injected, the hormone is ineffective in preventing their action.

The effect of cortisone on the course of hypertensive cardiovascular disease induced in the rat by DCA overdosage is not entirely clear. Friedman *et al.*, (1950) report that cortisone prevents the development of hy-

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pertension, but augments the renal lesions which that hormone produces. Masson *et al.* (1951) on the other hand, find that when cortisone is given together with DCA there is a more rapid onset of hypertension and a more marked degree of edema and proteinuria than when DCA alone is given. That a fundamental antagonism may exist between the action of glucocorticoids and mineralocorticoids on the cardiovascular system is indicated by the results of Woodbury *et al.* (1950) who found that adrenal cortical extract, which has a predominantly glucocorticoid action, tended to prevent the cardiac and renal hypertrophy which otherwise occurs in DCA-treated rats. This agrees with the observation of Perera and Pines (1949) that adrenal cortical extract prevents the elevation of blood pressure which DCA produces in hypertensive men. The antagonism between the two types of activity, as exemplified by cortisone and DCA respectively, is further evidenced by their opposite effects on the arthritic changes induced in the rat by local formalin injections (Coutu and Selye, 1950) and in the guinea pig by ascorbic acid deficient diets (Schaffenburg *et al.*, 1950).

In view of the fact that there is evidence to indicate that cortisone might, under certain circumstances at least, modify the course of hypertensive cardiovascular disease, it was decided to examine the effect of cortisone on the course of the syndrome which develops in parabiotic rats.

MATERIALS AND METHODS

Eleven pairs of rats in which one of the partners had developed hypertension, as a result of being placed in parabiosis, were studied. Postoperatively the pressure was measured once weekly in each of the pairs by the Sobin (1946) modification of the plethysmographic method originally devised by Williams, Harrison and Grollman (1939), on unanaesthetized animals. The arithmetic mean of four consecutive pressures, agreeing to within 5 mm. Hg was taken to represent the blood pressure of the animal in question; rats having pressures in excess of 150 mm. Hg were considered hypertensive.

In each case hypertension was considered established when pressures taken on two successive weeks each were higher than 150 mm. Hg and the second reading higher than the first. Hypertensions of varying degrees having been established, the rats were injected subcutaneously with 1 mg./day cortisone acetate (Cortone, Merck*). In a few instances (see Table 1) this dosage was later raised to 2 or 3 mg./day. Normotensive partners of hypertensive rats were not injected, but hypertensive parabionts were treated until it was felt that a full response had been achieved. The animals were then sacrificed and various tissues were removed for histologic examination.

RESULTS

Of the eleven hypertensive rats treated with cortisone, the blood pressure declined in nine. Eight became entirely normotensive, whereas the

*The authors are indebted to Dr. Augustus Gibson of Merck & Company for generous supplies of cortisone acetate.

ninth, in which the pressure had declined from 209 mm. Hg to 152 mm. Hg on therapy, had to be sacrificed because of the death of its parabiont. The blood pressure of normotensive parabionts was not affected by treatment of the hypertensive partners in any case; therefore no further consideration of them will be attempted. While the maximum period of treatment required to restore a normal blood pressure was 44 days, two responded within a week. In general long standing and severe hypertension

TABLE 1. THE EFFECT OF CORTISONE ON THE BLOOD PRESSURE OF HYPERTENSIVE PARABIOTIC RATS

	Before cortisone				After cortisone				
Pair No.	Hypertensive		No. of days hypertensive	Level of B.P. attained mm. Hg	Hypertensive		No. of days hypertensive	Dose mg./day	Final blood pressure mm. Hg
	From day*	To day			From day	To day			
1	25	36	9	194	36	45	9	1	121
2	17	30	13	225	30	56	26	1	126
3	10	25	15	228	25	40	15	1	96
4	17	24	7	209	24	38	14	1	152†
5	11	62	51	172	62	70	8	1	132
6	25	38	13	201	38	82	44	1→2	132
7	7	24	17	225	24	43	19	1→2	134
8	14	40	26	161	40	47	7	1	137
9	26	40	14	180	40	47	7	1	134
10	14	23	9	187	23	52	29	1→2	177
11	10	23	13	224	23	52	29	1→3	244

* Day of parabiosis.
† Death of partner on 38th day.

required more prolonged treatment than moderate hypertension of short duration, although this was not invariably so. Blood pressure was considered to have returned to normal levels when determinations made on two consecutive weeks each yielded values less than 150 mm. Hg; in general these were usually between 120 and 140 mm. Hg. Two of the hypertensive animals remained so despite an increase in the dosage of cortisone to 2 and later to 3 mg./day. In one of these the pressure became stabilized at about the pretreatment level; whereas it continued to rise in the other. The essential data are summarized in Table 1. The response of arterial pressure to cortisone is shown in Figs. 1 and 2.

The response to cortisone was often dramatic. One rat, hypertensive since the 12th day of parabiosis, developed a marked tic on the 25th day. This took the form of a spasmodic rotation of the head and shoulder girdle and was repeated two or three times each minute. The blood pressure was, at this time, 225 mm. Hg. Convulsive seizures of this type are due to cerebral vascular lesions resulting from hypertension, and are commonly encountered in hypertensive DCA-treated rats (Selye *et al.*, 1944). In the present instance the animal was emaciated and almost moribund. Although

any form of therapy seemed foredoomed to failure, cortisone treatment was initiated. Within a few days the tic had disappeared and the blood pressure began to decline. On the 19th day of treatment the arterial pressure was found to be 126 mm. Hg. Such rapid recoveries from hypertension were quite typical of cortisone-treated rats.

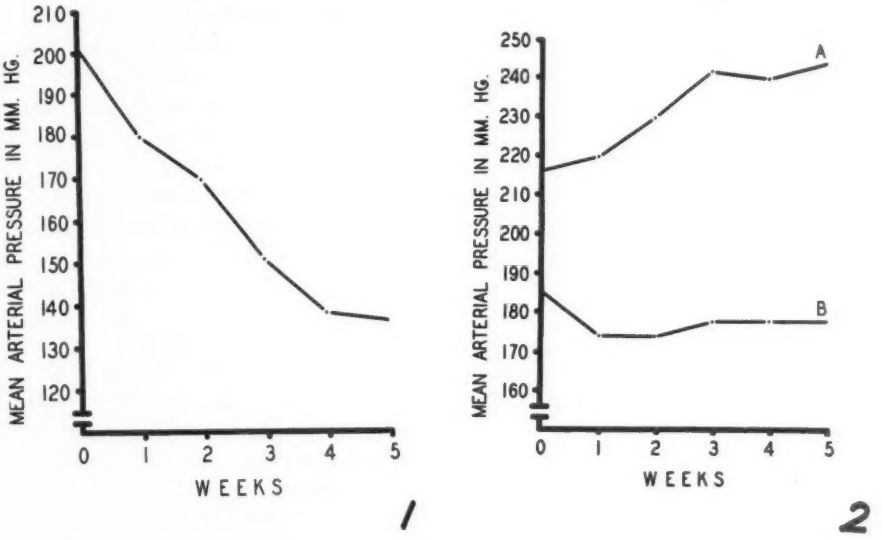

FIG. 1. Blood pressure in nine hypertensive rats following cortisone treatment. The dosage was, in each case, initially 1 mg./day, later raised to 2 mg./day in two instances.

FIG. 2. Two cases of hypertension not reversed by cortisone. Animal A developed, prior to treatment, severe periarteritis, still active at autopsy. Healed and less extensive periarteritic lesions were present, on histologic examination, in B. In each the initial dosage of 1 mg./day was raised to 2 mg. on the 10th day, in A this was increased to 3 mg./day on the 20th day.

MICROSCOPIC OBSERVATIONS

In our experience, parabiotic hypertension is associated with a high incidence of severe cardiovascular lesions. By contrast these were rarely observed in the present experimental series and then, with one exception, usually in the healed stage.

PANCREAS, MESENTERY AND HEART

Although periarteritis most commonly occurs in these areas in hypertensive rats, it was rarely observed in the present experimental series. Only three animals displayed such lesions. One of these was an animal in

which the blood pressure had reversed to normal under cortisone therapy, and here the prominent feature was repair. The remaining lesions were observed in the two animals which did not benefit from treatment. In the one whose blood pressure remained essentially unaffected by cortisone the lesions were healing. There was no evidence of the characteristic subendothelial hyalin deposition, and the vessel wall instead of being disrupted by an intensely cellular proliferating granuloma consisted of relatively acellular scar tissue. In the other, by contrast, the periarterial lesions were active, intensely proliferating, richly cellular and gave no evidence of having been affected by cortisone. The blood pressure had continued to rise in this animal despite an increase in the dosage of hormone to 2 mg./day in the second and 3 mg./day in the third week of treatment. This was the only animal in which severe periarteritis of the mesenteric vessels, detectable by palpation and confirmed by laparotomy, had developed before treatment was started.

KIDNEY

Glomerular engorgement, a specific effect of cortisone, was commonly observed in the hormone-treated rats. The changes characteristic of hypertension, consisting of arteriolar hypertrophy and sclerosis, glomerulosclerosis and tubular casts, were present in six of the eleven animals. In four, where hypertension had disappeared on treatment, the lesions were graded either "trace" or "+" on a scale on which "+++" represented maximal severity. For the most part the only evidence of renal damage was the presence of hyaline casts in a few of the tubules. The renal structure in the two rats whose arterial pressure remained elevated despite hormone treatment contrasted markedly. In the one in which the blood pressure had stabilized, the only indices of damage were a few tubular casts; whereas that in which the pressure had continued to rise despite treatment had the most severe structural changes. Hyalinized arterioles and glomeruli were the rule rather than the exception, tubular casts everywhere filled the tubular lumina and it was not possible to discover an area of normal appearance in the section.

DISCUSSION

In the majority of instances cortisone successfully halted the progress of the hypertension which had developed in parabiotic rats and caused the elevated pressure to return to normal. The results are thus in agreement with the evidence previously cited which indicated that cortisone might be a useful therapeutic agent in certain types of hypertensive disease.

It would appear that a reversal of hypertension may be achieved even in the presence of vascular lesions, since these were present, usually in an

arrested and healed form, in many of the animals which had previously been hypertensive. However the treatment was most rapidly effective when hypertension was of recent onset and mild in degree. Severe and prolonged hypertension required, in general, a longer period of treatment to bring about a restoration of normal blood pressure levels. Two animals in this category failed to respond to treatment with a decline in pressure, and it is interesting and perhaps significant that both of these had developed periarteritis prior to treatment.

When the vascular lesions were extremely severe, as in the hypertensive partner of pair No. 11, which had developed severe mesenteric periarteritis before treatment was begun, the blood pressure did not decline upon treatment. On the other hand the presence of healed lesions in cortisone-treated rats which did respond, in particular the one which had developed a tic, indicates that the presence of vascular lesions does not necessarily imply an irreversible hypertension. Whether or not an elevated blood pressure may be abolished by cortisone may well depend upon the extent and degree of vascular damage which has developed before treatment is instigated: the degree of renal damage may be especially determinant.

It is our view that the spontaneous hypertension which develops in parabiotic rats is the result of a metabolic disorder. Ultimately, if the condition proceeds unchecked, vascular damage results as a consequence of the hypertension. Cortisone, if given before the vascular damage has become excessive, interferes with the maintenance of an elevated blood pressure. This prevents further vascular damage and permits the healing of lesions which have developed. If vascular involvement has proceeded to an extent which results in a generalized increase in peripheral resistance, perhaps involving activation of the renal pressor mechanism, hypertension is refractory to treatment.

SUMMARY

The hypertension which spontaneously develops in parabiotic rats may be abolished, in the majority of cases, by cortisone treatment.

The response is most rapid when the hypertension is mild in degree and recent in onset.

Periarteritic lesions, if not too extensive and associated with a refractory hypertension, rapidly heal under cortisone therapy.

The evidence is suggestive, although not conclusive, that hypertension in animals with severe coexistant arterial damage is not amenable to cortisone therapy.

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THE EFFECTS OF DOSAGE, AND OF ESTROGEN, ANDROGEN OR SALICYLATE ADMINISTRATION ON THE DEGREE OF BODY TEMPERATURE ELEVATION INDUCED BY PROGESTERONE

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INTRODUCTION

THE literature concerning the thermogenic effect of progesterone has by now attained sizeable proportions (see the review by Palmer, 1949), yet with the exception of the study of Isreal and Schneller (1950), and those reported in abstract by Perlman (1948), and Nieburgs and Greenblatt (1948), practically no attempt has been made to study either the quantitative aspects of this effect, or the essential physiologic processes involved. Isreal and Schneller (1950) and Perlman (1948) indicate that the rise in body temperature is related to the dose of progesterone. Nieburgs and Greenblatt (1948) state that in rats the effect is present in the absence of ovaries, adrenals and thyroids. In the main, concomitant administration of estrogens does not alter the thermogenic effect of progesterone (Buxton and Atkinson, 1948; Davis and Fugo, 1948; Isreal and Schneller, 1950) although estrogens alone are almost uniformly claimed to have a temperature depressing effect. However, Isreal and Schneller (1950) have shown a temporary depressant effect of single doses of estrogens (estradiol benzoate, 1.0 and 1.5 mgs.) on the elevated temperature of women in the postovulatory phase of a normal menstrual cycle. While these doses were undoubtedly very high, those used by Magallan and Masters (1950) to inhibit the progesterone induced body temperature rise were considerably smaller and conceivably closer to ranges of estrogen: progesterone ratios probably existing in the body (1.0 mg. estradiol benzoate two times weekly plus 20.0 mgs. progesterone per day). Androgens are conceded to be without effect on the elevated temperature induced by progesterone (Davis and Fugo, 1948; Magallan and Masters, 1950), although by themselves they have been variously claimed to lower the body temperature of rats (Nieburgs and Greenblatt, 1948), or to reduce the amount of daily variation in body temperature in human subjects (Magallan and Masters, 1950).

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Buxton and Engle (1950), from another standpoint altogether, have raised a question which bears on the thermogenic property of progesterone. Their study has indicated that in some women as much as three days may elapse between ovulation, with concomitant corpus luteum formation, and the characteristic elevation of the body temperature at mid-cycle, and in others (although here the time element is completely unknown) that the rise in body temperature may occur before ovulation. The quantitative aspects of body temperature response to progesterone are obviously of importance here. Without them, there is little basis on which to evaluate the postulate that a normal corpus luteum as much as three days old will secrete too little progesterone to elevate the body temperature at all.

To what extent the cyclic body temperature curve in women is an accurate indicator of the time, as well as of the occurrence of ovulation is an important question. Studies on the physiology of human ovulation have been seriously hampered by the lack of criteria with which to determine or predict the time of ovulation. The basal body temperature curve has the distinct advantage over other methods in that a minimum of time and equipment is required. Here, then also, we must be aware of the real limitations of this method, and of the extent to which the body temperature changes adequately reflect the physiologic changes taking place in the ovary.

The studies reported here, while not achieving the final answers to all these questions, have been carried out in an attempt to improve our understanding of the nature of the body temperature response to progesterone. The factors limiting their completeness are on the one hand, those invariably associated with the use of human subjects for physiologic studies, and on the other, those related to the setting up of techniques for the taking, reading and recording of basal body temperatures on fairly large numbers of human subjects under standard conditions. Further work along the main lines of this study will be reported on to the extent that these limitations can be obviated.

METHODS AND MATERIALS

The subjects were 28 male inmates of a home for the feeble-minded. Their mental status corresponded to the classification called feeble-minded (as distinct from *imbecile* or *idiot*) by Tredgold (1947). Ten subjects ranged in age from 15–18 years and the remainder were adults 29–39 years old. All were "physiologically normal," as far as could be ascertained. The groups of adolescent and adult subjects were each housed in separate dormitories; their times of arising and going to bed and their diet and routine of daily activities were practically constant from day to day. Body temperatures were obtained per rectum (5 minutes) with ordinary clinical thermometers. Individual thermometers were used for each subject, and with very little variation, the time of taking temperatures was the same each day for each subject. All temperatures were taken between 5:30 A.M. and 6:30 A.M., with the subjects in bed, and read later in the day to the nearest

0.10° F. Only one person took and recorded all temperatures. In over 100 trials, in which his thermometer readings were checked by three highly trained individuals, he was never found to be in error.

In general, these studies were directed toward 1) the relation between progesterone dose and the extent of the body temperature elevation; 2) the effects of other steroids, such as testosterone and estradiol, and 3) the effect of salicylate antipyretic agents, on the elevated temperature induced by progesterone.

Progesterone[1] was used in a dosage ranging from 1.0 to 50.0 mgs. per day for periods of from one to 26 days. The route of administration was in all cases intramuscular (deltoid or gluteal region) and the volume per injection was in most cases between 0.4 and 1.0 cc. The solvent in all cases was sesame oil. In the studies involving concomitant testosterone[1] and progesterone administration the two hormone solutions were mixed in the proper proportions so that one injection carried the desired amounts of each hormone. Where estradiol[1] was given along with progesterone, however, each hormone was given by separate injection. Salicylates were either aspirin, or sodium salicylate, the former in five grain tablets, and the latter in five grain enteric coated tablets. They were given at bedtime, and in each series in which they were tried, for three to five consecutive days.

Except where noted, the mean temperature of each subject was calculated for periods of from three to five days, and compared with the mean temperature during equivalent periods before or after the particular one under study. Group means were calculated from the individual means, but no attempt has been made to subject the differences noted to statistical analysis.

Oil injection control studies were not included for two reasons. Studies by other workers have sufficiently established the reality of the thermogenic effect of progesterone. Even if this were not so, however, the fact that the very small doses of progesterone (1.0 mgs.) as well as testosterone propionate were without effect on the body temperature (see below) would serve in place of oil injection controls.

RESULTS

I. *Quantitative aspects of the thermogenic property of progesterone*

A. *Effects of single doses.* The relation between the extent and duration of the rise in body temperature following a single injection of progesterone is best seen by means of a scatter graph (Figure 1). In this study, injections of 1.0, 10.0, 25.0, and 50.0 mgs. were made in 10, 5, 9, and 5 men respectively. In several cases one man received more than one dose, but a period of from four to seven days elapsed between the two injections. The results are expressed as the difference, in 0.10° F., between the mean control temperature for a period of five days, and the temperature on each of four to seven consecutive days following the injection. A clear-cut relationship between the extent of the rise and the size of the dose was not seen, but it was fairly apparent that a 1.0 mg. dose was practically inef-

[1] The supplies of progesterone, estradiol (micropellets and estradiol dipropionate), and testosterone propionate used in this study were donated through the courtesy of Dr. Edward Henderson, of the Schering Corporation, Bloomfield, New Jersey.

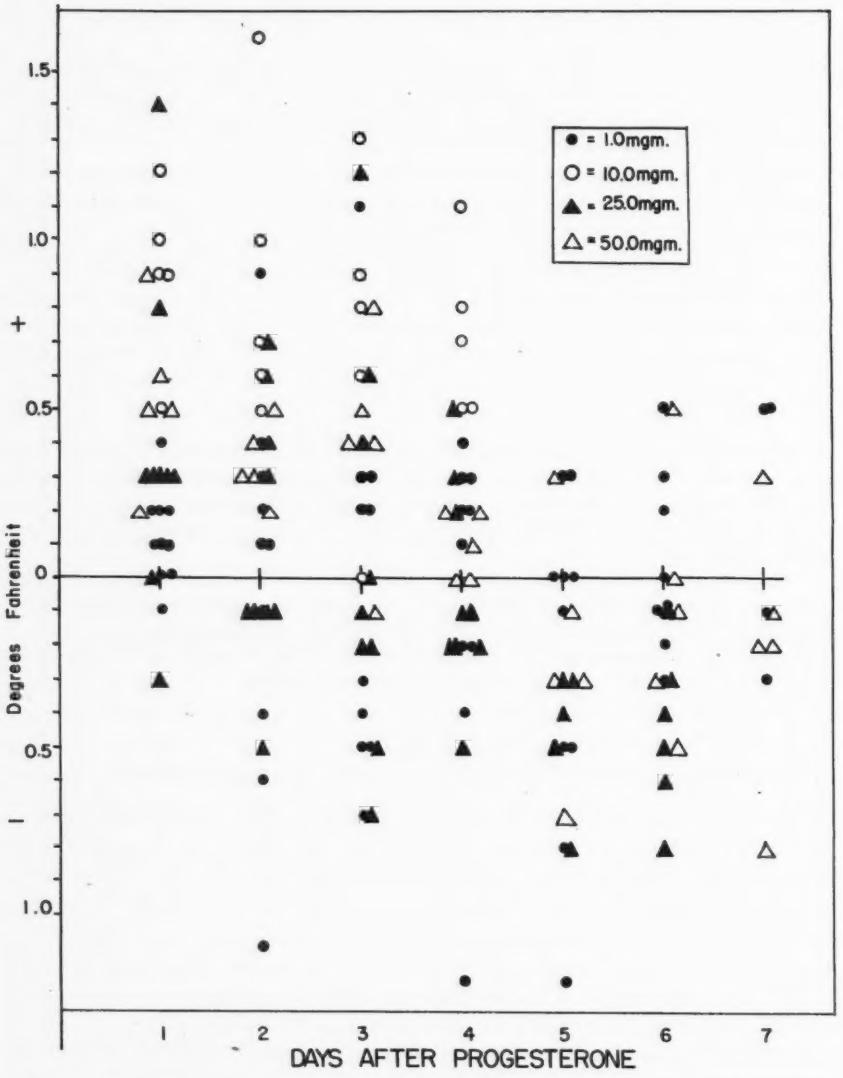

FIG. 1. *The body temperature response to single doses of progesterone.* The body temperatures following a single injection of progesterone are shown as the differences between the control temperatures and the temperatures on each of seven days following the injection.

fective in producing a significant rise in body temperature. The other dosages induced significant elevations in temperature that usually lasted for not more than two to three days. By the fourth day, practically all temperatures were fluctuating around the control level.

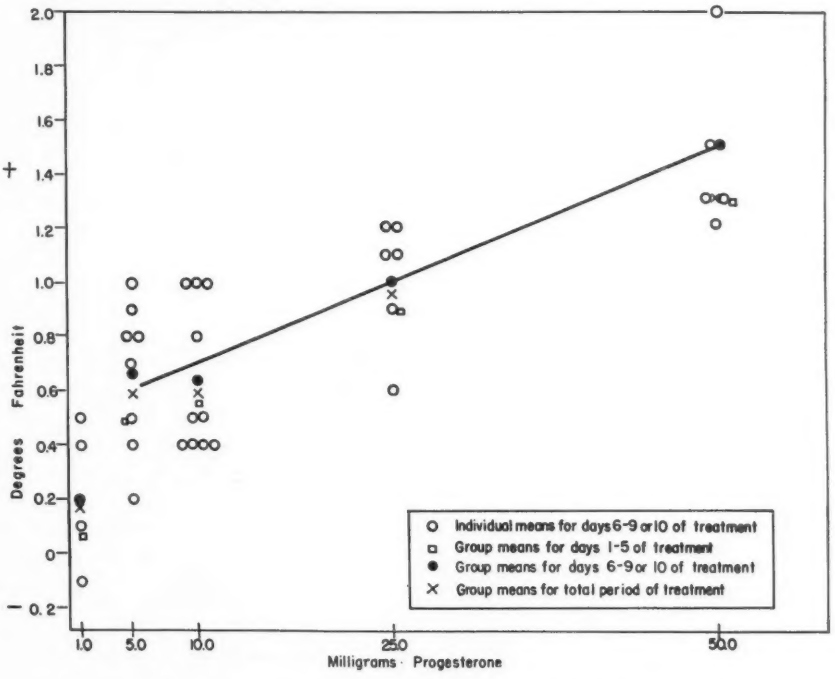

FIG. 2. *The relation between the dose of progesterone and the body temperature response after multiple injections.* The results are expressed as the differences between the control temperatures and the mean temperatures during the first five days of treatment, the second five (or four) days of treatment and the total period of treatment. Injections were made for 10 days in the groups receiving 1.0, 5.0, or 10.0 mgs., and for nine days in the groups receiving 25.0 or 50.0 mgs. of progesterone.

B. *Effects of multiple doses.* Progesterone in doses of 1.0, 5.0, or 10.0 mgs. was injected daily for a total of 10 days into 4, 8, and 10 subjects, and in doses of 25.0 or 50.0 mgs. daily for nine days into 6 and 5 subjects, respectively. The results are expressed as the difference between the mean body temperature of each subject for a period of five days before the period of injection, and the mean body temperature during the first five days, the next four or five days, and the total period of progesterone administration (Figure 2).

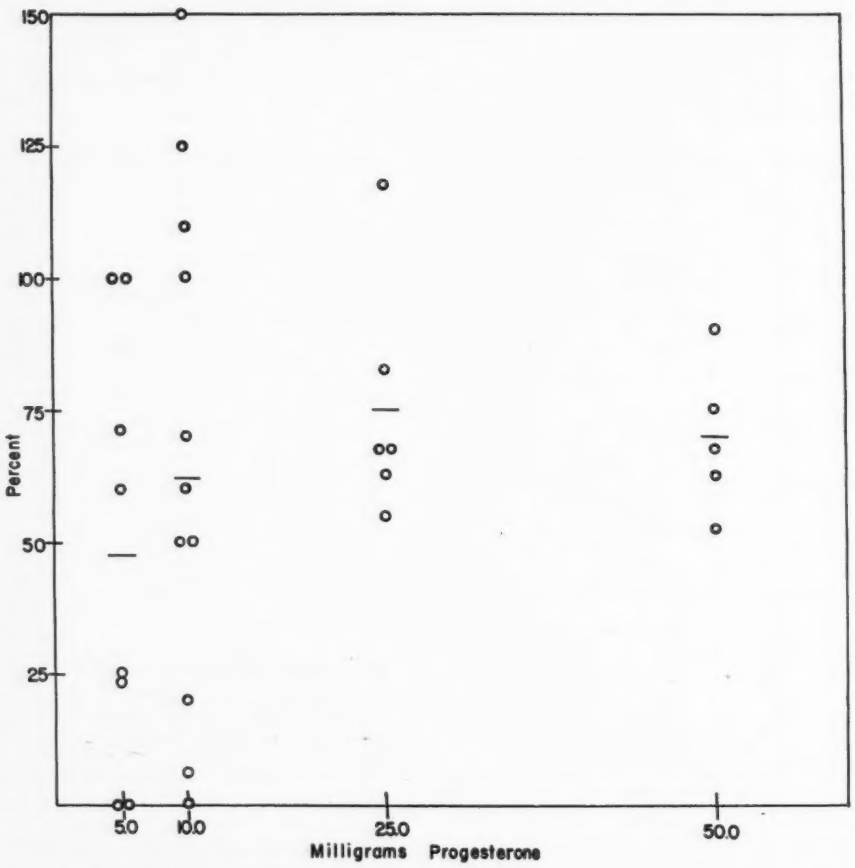

FIG. 3. *The difference between the mean control temperature and the temperature on the day after the first injection of progesterone expressed as the percentage of the difference between the mean control temperature and the mean temperature during the second five or four days of treatment.* The individual values are shown by the open circles, the mean value for the group by the short horizontal line.

The 1.0 mg. dose produced no obvious change in the mean body temperature. Doses of 5.0 mgs. or more led to significant elevations in body temperature, and the differences between the group means and the mean control temperatures for the particular groups was directly related to the size of the daily dose between 10.0 and 50.0 mgs. The slope of the curve is not steep, however, and the amount of variation is obviously large.

The differences between the mean body temperatures of the various

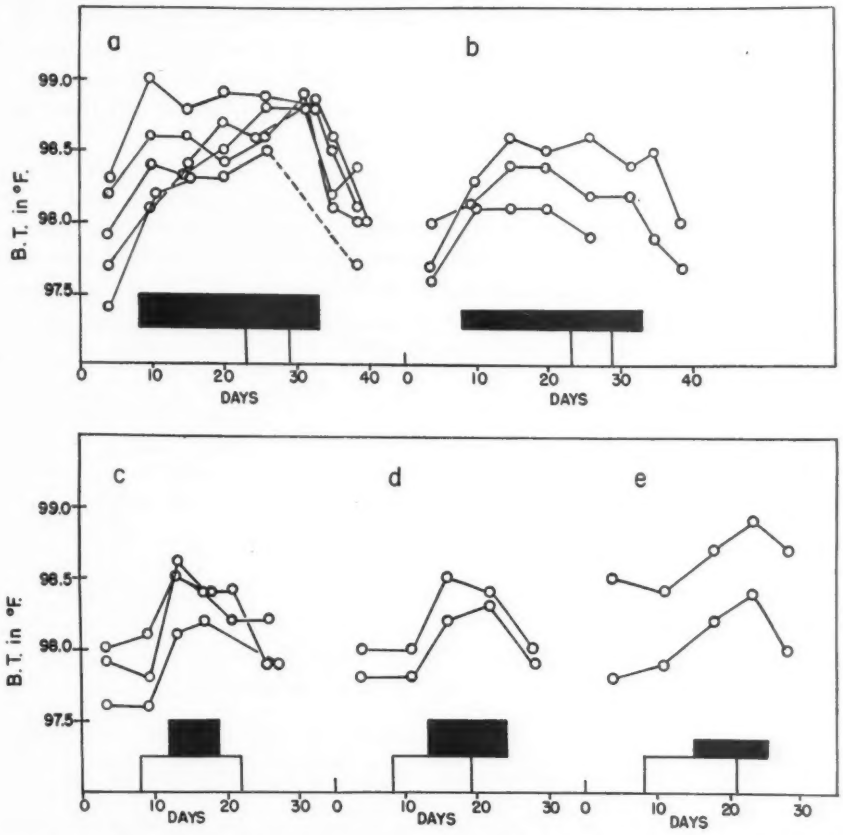

FIG. 4. *The lack of effect of testosterone on the thermogenic effect of progesterone.* Each curve represents the body temperature of a single subject for periods of up to 40 days. For convenience, these temperatures are expressed as the means for periods of from three to six days. Progesterone treatment is indicated by the shaded, testosterone propionate treatment by the open horizontal bars. Dosage of testosterone propionate was in all cases 10.0 mgs. per day. Progesterone dosage was 10.0 mgs. per day in all except (b) and (e), where it was 5.0 mgs. per day.

dose groups were seen to be due to establishment of plateaus at different levels in each group, rather than to differences in the extent of *continued* daily temperature elevations. In almost all cases these plateaus were established by about the fifth day of treatment or earlier, but, allowing for the small number of data involved, appeared to be established at different initial rates. A rough indication of this is seen in Figure 3, in which the difference between the mean control body temperature and the temperature

on the day after the first injection is expressed as the percentage of the difference between the mean control temperature and the mean temperature for the second five days of treatment. Apparently the higher doses of progesterone led not to a more *rapid*, but to a more *uniform* attainment of this plateau than did the lower doses.

II. A. *Effect of testosterone*

The following experiments were carried out. a) Ten mgs. of testosterone propionate were injected per day for seven days into each of three men receiving 5.0 mgs., and into each of five men receiving 10.0 mgs. of proges-

TABLE 2. THE LACK OF EFFECT OF ESTROGEN ON THE THERMOGENIC EFFECT OF PROGESTERONE

Subject	Daily dose progesterone	Mean body temperatures								
		Before progesterone	Progesterone alone		P'sterone plus estrogen				After progesterone	
					Estradiol micropellets[1]		Estradiol dipropionate[2]			
	(mgs.)	(5)*	(5)*	(4)*	(3)*	(3)*	(3)*	(3)*	(5)*	(5)*
JA	10.0	97.9	98.6	98.9	98.8	98.6	98.6	98.4	98.1	98.4
GM	10.0	97.8	98.6	98.8	98.6	98.8	98.7	98.7	98.4	98.3†
TS[3]	10.0	97.8	98.7	98.6	99.0	98.6	98.5	98.6	98.2	97.9
FP	5.0	97.4	98.4	98.5	98.2	98.3	98.6	98.2	98.2	98.1
RL	5.0	97.5	98.0	98.1	98.2	97.7	98.0	98.0	97.8	97.6
AS	5.0	97.8	98.3	98.6	98.1	98.4	98.2	98.5	97.9	97.8

* The number of days of recorded body temperatures from which the mean temperatures noted were calculated. In all cases these were consecutive days.

† This mean is derived from only two days' temperatures.

[1] Two injections of the micropellets, 1.0 mg. each, 3 days apart. The temperatures noted were the means of the first, second and third days temperatures after each injection. There was no consistent difference between the temperatures on the day following each injection and those on the second and third days after each injection.

[2] The estrogen was given in oil, 1.0 mg. per subject per day, for a total of 6 days. The first injection was given on the 4th day after the last micropellet injection.

[3] A cretin. All the others in this experiment, as noted in the text, were high grade feeble-minded people.

terone per day for 26 days. The androgen was injected starting on the 16th day of the progesterone series. b) Two men received a series of testosterone propionate injections (10.0 mgs. per day) for 11 and 12 days, respectively. On day five of this series in one subject and on day six in the other, progesterone was started (10.0 mgs. per day) and continued for 12 days. In another group of three men, 17 daily injections of testosterone propionate were made (10.0 mgs. per day), with progesterone (10.0 mgs. per day) given on days five through 12 of the androgen series. c) Finally, two cretins were treated very much as was the first group described under (b) of this paragraph. The results (Figure 4a, b, c, d, e,) show no effect of the androgen on the thermogenic effect of progesterone.

B. *Effect of estradiol.* Two groups of three men each were injected with 5.0 or 10.0 mgs. of progesterone, respectively, daily for 21 days. On the 10th and 13th day of progesterone administration, 1.0 mg. of estradiol micropellets in saline suspension was injected per subject, and on the 16th through the 21st days, each subject received 1.0 mg. of estradiol dipropionate per day. The results are shown in Table 1. Here also, no effect of the estrogen on the progesterone induced body temperature rise was seen.

III. *Salicylates on the elevated temperatures induced by progesterone*

a) Five subjects were injected for 15 days with 5.0 mgs. progesterone per day, and were given 20 grains of aspirin per day for four days starting on the seventh day of progesterone administration. b) Four subjects were injected for 15 days with 10.0 mgs. of progesterone per day, and were given 20 grains of aspirin and 15 grains of sodium salicylate per day for four days starting on the seventh day of progesterone administration. c) Six subjects (adolescents) were injected for 22–25 days with 10.0 mgs. progesterone per day and were given 20 grains of aspirin per day for four days starting on days three to six of progesterone administration. Two were again given the same dose of aspirin for three days, and four for five days, starting on day 15 of the progesterone administration. d) Three men were injected for nine to 12 days with 25.0 mgs. of progesterone per day and were given 20 grains of aspirin daily for four days starting on the second to fifth day of progesterone administration. Two men treated as in (d) were continued on 25.0 mgs. of progesterone for 17 days, and given 20 grains of aspirin daily for four days starting on the ninth day of this second course of progesterone administration.

In the (a) and (b) groups no effect of the salicylates was seen. In the (d) group, although there was no temperature depression during the period of salicylate administration, all the men showed a small mean rise (0.1 to 0.4° F.) for the period of four days following the cessation of salicylates. In the (c) group some slightly more definite, but still far from conclusive indications of an effect were seen. Thus, during the first two days of progesterone administration the mean rise was 0.65° F. (range: 0.3 to 0.9° F.). During the period of aspirin administration, the mean group temperature was lowered 0.3° F., although the individual changes were quite varied (+0.1°, 0.0°, −0.1°, −0.2°, −0.6°, and −0.9°). In the first three days after the cessation of aspirin administration, the temperatures tended to rise again (mean group change was +0.1° F.), and this was continued during the next four days, when the mean group temperature rose 0.25° F. During the second course of aspirin administration, all but one boy showed a slight lowering of the temperature (+0.1°, −0.1°, −0.1°, −0.3°, −0.4°, and −0.4°), but in only two of them did the temperature rise at all when the aspirin was stopped. These two boys also showed the most marked depression of temperature during the first course of aspirin, and the most marked rise, when the aspirin was discontinued.

DISCUSSION

Magallan and Masters (1950) interpreted their failure to obtain a body temperature rise with progesterone in subjects receiving concomitant small doses of estrogens as an inhibition of the progesterone effect by

greater than critical levels of estrogens. However, the estrogen/progesterone ratio in their studies was only 1/70 (1.0 mg. estradiol benzoate twice per week and 20.0 mgs. progesterone per day). In our own study, we saw no inhibition of the progesterone effect with ratios as high as 1/5 and 1/10. The subjects in Magallan and Masters study were aged females, while ours were young adult males. The difference in response may be due to a peculiarity of old age, for our own findings, plus those of others (Davis and Fugo, 1948; Isreal and Schneller, 1950), make it difficult to evade the conclusion that the estrogens are relatively ineffective in inhibiting the thermogenic effect of progesterone. Even less effective are the androgens. The estrogens alone, and less so, the androgens, do have a temperature depressing effect. Since progesterone exhibits its thermogenic properties in their presence, it is probable that these steroids act on different elements of the heat regulating mechanisms. As far as body temperature is concerned, the estrogens may be safely assumed to affect it through their cutaneous vascular effects, and, if we may follow our argument through logically, then progesterone may very well act centrally. The salicylate studies have given us a hint that this may be correct, but studies involving the determination of the time element in the body temperature response to progesterone are needed in order to see the salicylate effect, if it is a real one, more clearly.

In Buxton and Engle's (1950) study, women who were admitted to the hospital several days before the expected date of pelvic surgery had their basal temperatures recorded daily. On that day on which the temperature rose significantly, surgery was undertaken, the ovaries examined, and either the ripest follicle, if no ovulation had occurred, or the freshly formed corpus luteum was removed. Endometrium was also obtained by curettage or hysterectomy. Six patients were found to have corpora lutea 24 or less hours old, six had not yet ovulated, while the remaining six exhibited corpora lutea 36 hours (two patients), 48 hours (two patients), and 72 hours (two patients) old. Considerable doubt as to the validity of the BBT curve as an indicator of the time of ovulation was raised as a result of this study.

Other studies (Everett, 1948; Fraps and Dury, 1943; Rothchild and Fraps, 1949; and Pfeiffer, 1950) have indicated that progesterone can induce ovulation. The preovulatory rise in body temperature in some of the patients in Buxton and Engle's study may therefore be explainable on this basis. More disturbing, however, is their finding that a corpus luteum as old as 72 hours may secrete too little progesterone to elevate the body temperature but enough to bring about some endometrial changes. That a rise in body temperature occurs within at least 24 hours after the parenteral administration of progesterone is inescapable, and equally clear is the fact that as little as 5.0 mgs. of progesterone can elicit this effect. The

amounts of progesterone required to initiate progestational changes in the human endometrium are much greater than this (Bradbury, Brown and Gray, 1950). We would therefore consider seriously the possibility that these discrepancies between the times of ovulation and body temperature rise in some of Buxton and Engle's patients may be due to the *exceptional circumstances of hospitalization for elective surgery*, which in these patients prevented them from reacting with a body temperature rise to the progesterone secreted by their ovaries. Whether an equal percentage of women in the general population would also fail to react to progesterone under similar circumstances is a question as interesting as it is unanswerable at present.

CONCLUSIONS

There is a direct quantitative relationship between the amount of progesterone administered and the extent of the body temperature rise.

The body temperature response to progesterone is rapid, occurring within at least 24 hours after the first injection, and is fairly sensitive, since it occurs with as little as 5.0 mgs. per day.

The body temperature elevation tends to remain constant after the first few days of progesterone treatment. The attainment of the plateau is more uniform with higher doses (25.0–50.0 mgs.) than with lower ones (5.0–10.0 mgs. per day).

Neither estrogens (estradiol dipropionate) nor androgens (testosterone propionate) affect the thermogenic property of administered progesterone.

Salicylates, under the circumstances of these experiments, did not have a uniform inhibiting effect on the thermogenic property of progesterone, but with some doses in some individuals, evidence for such an inhibiting effect was seen, thus lending some weight to the possibility that the thermogenic effect of progesterone is mediated through a hypothalamic center.

SUMMARY

The thermogenic properties of progesterone were studied in a group of 28 male inmates, both adolescent and adult, of a home for the feebleminded. Single doses of progesterone between 5.0 and 25.0 mgs. were followed by body temperature elevations lasting from one to 5 days but without clear-cut evidence of a quantitative relation between the dose and response. Such a relationship was seen, however, when multiple daily doses of progesterone were used. Progesterone administered during a course of testosterone propionate treatment exerted its typical thermogenic effect, nor was its effect modified when testosterone treatment was added to a course of progesterone injections. Estradiol administered either as the water suspension of the free hormone, or the oil solution of the dipropio-

nate in a dose of 1.0 mg. per day to subjects receiving either 5.0 or 10.0 mgs. of progesterone per day did not depress the response to the progesterone. In some of the adolescent subjects fairly good evidence for a depression of the body temperature elevation induced by progesterone by concomitant administration of salicylates was seen, but the effect was not uniform enough to postulate definitely a contrary action of the two drugs on the same heat-regulating mechanism.

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